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ANTIGENIC DIFFERENCES IN STRAINS OF EPIDEMIC INFLUENZA VIRUS: I. CROSS-NEUTRALIZATION TESTS IN MICE.

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IN a previous report it was shown that although the PR8 and Phila strains of epidemic influenza virus are rather closely related, there exist between them definite antigenic differences which can be demonstrated by appropriate methods (Magill and Francis, 1936). Earlier work had shown that rabbits repeatedly inoculated with the PR8 strain of virus produced antibodies against both the PR8 and Phila strains, and also against the virus of swine influenza (Francis and Shope, 1936). It was thought that the rabbit, being non-susceptible to infection with influenza virus, might reflect slight differences in the antigenic structure more readily than a susceptible animal. Moreover, it seemed reasonable to believe that if antigenic differences existed between strains of influenza virus, they might be best demonstrated early in the course of antibody response of an animal which had received but a single inoculation. Accordingly, young adult rabbits were inoculated with one dose of virus intraperitoneally, and the serum obtained nine to fifteen days later was used in mouse-protection tests against the homologous and heterologous strains of virus. The results showed clearly that serum prepared against the PR8 strain of virus protected mice against that strain but not against the Phila strain, while the Phila antiserum protected against the Phila and to a lesser extent against the PR8 strain. It was also shown that antiserum prepared as the result of vaccination with swine influenza virus furnished protection to mice against that virus and to some degree against the PR8 strain of epidemic influenza virus, but not against the Phila strain. Subsequently Burnet (1937) and Stuart-Harris, Andrewes and Smith (1938) demonstrated even greater differences among other strains of influenza virus. Burnet tested the effect of

ferret antisera on strains of virus propagated on the chorio-allantoic membrane of the chick, while Stuart-Harris, Andrewes and Smith tested the neutralizing capacities of ferret sera on strains of mouse-passage virus. It is to be noted that in this laboratory ferret sera have not brought out the sharp differences among strains that can be demonstrated by means of rabbit sera.

Since the original report a considerable number of new strains of virus have been isolated and still more have been obtained from other laboratories, so that there has been opportunity for study of the antigenic relationships in 23 strains of epidemic influenza virus isolated during the past several years in various parts of the world and one strain of swine influenza virus. These studies have shown that some of the strains differ much more in their serological behaviour than do the PR8 and Phila strains, while still other strains seem very closely related to one another. The purpose of this report is to present the results of such studies and to discuss their significance.

METHODS.

Preparation of serum.—Healthy young adult rabbits were given intraperitoneally a single injection of virus in the form of a saline suspension of lungs of mice infected with a given strain. The suspensions varied in strength from 1 to 10 per cent., so that rabbits immunized with different strains received approximately the same amount of virus. Blood was obtained from the marginal vein of the ear or by cardiac puncture 8 days after inoculation, the serum separated and kept in a refrigerator without preservative. Under such conditions the sera produced against the greater number of virus strains were quite uniform in their capacity to protect mice against infection with the homologous strains of virus.

Recognizing the possibility of individual variations in the antibody response of different animals, an effort was made to establish a standard for sera by using only those which completely protected mice from pulmonary lesions when inoculated intranasally in a dilution of 1 : 40 with the homologous strain of tissue culture virus of standard strength. This proved not to be uniformly practicable, for with 6 of the strains it was impossible to prepare sera sufficiently potent to meet such requirements. In the results to be reported the antisera prepared against all but these 6 strains (Phila, Miss 4–5, Mo 5A, NY2, Gatenby and swine) protected mice against pulmonary lesions in dilution of 1 : 40, and against death in dilution of 1 : 160, when tested against the homologous strain of virus.

Virus.—Strains of virus which were well adapted to growth in tissue culture medium were used to test the protective capacity of the various antisera. The tissue culture technique is essentially the same as that previously described (Li and Rivers, 1930 ; Francis and Magill, 1935), except that for most of the strains it has been found satisfactory to substitute 0·85 per cent. NaCl solution for Tyrode solution as the medium in which the minced chick embryo cells are suspended. Most strains maintained in such cultures are quite uniform in infectiousness for mice, containing between 100 and 1,000 lethal doses of virus for each 0·05 c.c. of culture. There were, however, several cultures, particularly the WS and Melbourne strains, which consistently contained 10 to 100

times that number of lethal doses. Occasionally the titre of a strain, for the period of a transfer or two, might fall below 100 lethal doses. These variations rendered it essential that the various strains be standardized as to infectiousness for mice.

At the end of 48 hours' incubation the virus content of a portion of each lot of culture was determined by the intranasal inoculation of mice with 0·05 c.c. amounts of decimal dilutions. The remainder of each lot of culture was frozen at — 80° C. in alcohol-CO_2 ice mixture (Turner, 1938) and kept under these conditions until needed. Immediately before use the frozen cultures were rapidly thawed by pulverizing the solid material in a mortar, and the liquid culture was then centrifuged at 2000 r.p.m. for 15 minutes to remove the remnants of the chick embryo cells. Cultures containing more than 1000 lethal doses per unit of volume were diluted so that each inoculation would contain between 100 and 1000 lethal doses. Cultures containing less than 100 lethal or 10,000 infectious doses were discarded. Cultures preserved and handled as described retain unchanged their infectiousness for mice. It is important, however, that the frozen cultures be thawed rapidly, and not be permitted to stand at room temperature for prolonged intervals.

Neutralization or mouse-protection test.—The various rabbit antisera were tested in five final dilutions of 1 : 2, 1 : 5, 1 : 10, 1 : 20, and 1 : 40. Equal parts of the serum dilution and centrifuged tissue culture virus of standard strength were mixed and incubated at 37° C. After 30 minutes' incubation 0·05 c.c. of the serum-virus mixture was given intranasally to each of a group of three lightly anæsthetized Swiss mice 5 to 6 weeks old. The mice were observed for 10 days, all fatalities were recorded, and on the 10th day surviving mice were autopsied and the degree of pulmonary involvement noted. Thus the protective titre of a serum could be determined by either of two end-points : (1) the titre of serum which protects mice from death due to influenza virus for a period of 10 days ; and (2) the titre of serum which prevents development of pulmonary lesions in mice. At first appearance it might seem that the latter method is the more rigid, but a distinct parallelism exists between the two criteria.

RESULTS.

Results of Cross-Neutralization Tests.

The results of the present study not only confirm the previous findings of the existence of antigenic differences between strains of influenza virus, but show quite definitely that differences between some strains are much more marked than those originally reported between PR8 and Phila strains. Furthermore, the results indicate that although differences can be readily demonstrated between some strains, there are other strains which, under the conditions of the experiments, seem to be identical. When considering these differences and similarities it is well to recognize that each strain has two distinct characteristics : (1) The ability to imprint its characteristic structure on the serological mechanism of the rabbit in such a way as to evoke the production of a peculiar antibody complex ; and (2) the relative ability of the strain to infect

mice in the presence of its own antiserum as well as in the presence of antisera prepared against other strains. The former characteristic may be termed its *antigenicity*, the latter, for want of a better term, its *aggressiveness*.

The results are summarized in Chart I. In the vertical column to the left are listed the antisera prepared against the various strains of virus. At the head of each of the other vertical columns is given the strain of tissue culture virus against which the protective capacity for mice of the various sera was tested. Each space is divided vertically into five divisions indicating the dilutions of serum (1 : 2 to 1 : 40). Black areas denote that mice which received the respective dilution of serum with virus died. Shaded areas signify that the mice survived, but when autopsied showed varying degrees of pulmonary involvement. Clear areas indicate that mice not only survived, but showed no pulmonary involvement. It is obvious that the chart can be read in either of two ways, horizontally or vertically. With the former the information concerns that characteristic of the strain which we have called its antigenicity. Vertical reading concerns the strain's aggressiveness.

Source of Strains of Epidemic Influenza Virus.

Strain of virus.	Origin.	Date of isolation.
PR8	Puerto Rico	1934
Phila	Philadelphia	1935
Alaska	Alaska	1935
NY2	New York City	1936
Mo 5A	Missouri	1936
Miss 4–5	Mississippi	1936
Patnode	New York City	1936–37
Henry	,,	,,
Webb	,,	,,
Bl 5	,,	,,
TF	,,	,,
Bkl 15	,,	,,
Bkl 1	,,	,,
Smithb	,,	,,
LV	Letchworth Village, N.Y.	,,
Fla 12	Florida	,,
Melbourne*	Australia	1935
Bud C12*	Budapest, Hungary	1937
Talmey*	England	1937
Gatenby*	,,	1937
BH*	,,	1935
WS*	,,	1933
Phila 37–7*	Philadelphia	1936–37
Swine virus*	Iowa	1931

* The Talmey, Gatenby, BH and WS strains were obtained from Dr. C. H. Andrewes of London, England; the Melbourne strain from Dr. F. M. Burnet of Melbourne, Australia; the Bud C12 strain from Dr. R. M. Taylor of Budapest, Hungary; the Phila 37–7 strain from Dr. J. Stokes, Jun., of Philadelphia, Pa.; and strain 15 of the swine influenza virus from Dr. R. E. Shope of Princeton, N.J., to all of whom we are greatly indebted.

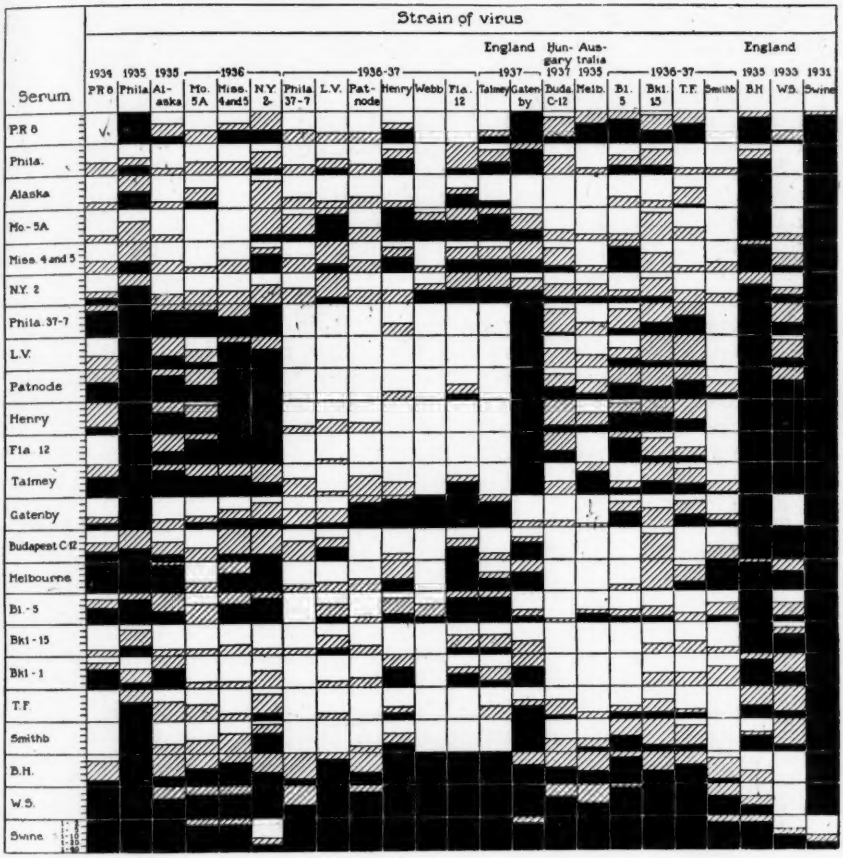

CHART I.—Cross-neutralization tests in mice with strains of influenza virus.

Considering the various strains with regard to both these characteristics it is possible to list them in several fairly well-defined groups, as follows :

1.	*2.*	*3.*	*4.*	*5.*	*6.*
Phila 37–7	Bkl 15	PR8	Mo 5A	WS	BH
LV	Bkl 1	..	Miss 4–5	..	Swine
Patnode	TF	..	NY2	..	..
Henry	Bud C12	..	Alaska	..	..
Webb	Melbourne	..	*4A*	..	..
Fla 12	Bl 5?	..	Phila	..	..
Talmey	..	..	Gatenby	..	..
Smithb	..	..	..	..	..

The similarities among the members of the various groups are very striking, and yet each group exhibits differences from the others. The strains grouped under *1* are closely related, and are characteristic in that each strain readily produced antiserum of high titre for the homologous strain, and also for the other strains in that group. They were also fairly well neutralized by antisera prepared against many of the strains in other groups.

In marked contrast to those strains are the ones under heading *6* (BH and swine). These strains induced in rabbits antisera of poor protective capacity against practically all heterologous strains. On the other hand, neither was appreciably affected by antisera prepared against most of the other strains.

Between the extremes represented by groups under headings *1* and *6* are the strains listed under *4*. They are peculiar in that they elicited antisera which were relatively poor in protective antibodies for the homologous strains, yet exerted a very good neutralizing effect upon certain heterologous strains. An example of this is seen in the behaviour of the serum produced against the Phila strain. This serum showed only partial neutralization with the homologous strain of virus, while in dilution of 1 : 40 it completely protected against both the Webb and WS strains. Moreover, the strains listed under *4* were not neutralized to a high degree by any of the antisera prepared against strains of other groups.

The strains of Group *2* tend to represent a transitional series between *1* and *4*. The PR8 strain does not fit well in any of the groups, but seems to belong between *2* and *4*. The WS strain, while approaching the characteristics of the BH strain in the type of antiserum produced, was neutralized to high titre by antisera which had little effect upon the BH strain.

It is readily apparent that sharp classifications are extremely difficult. While rough groupings may be made, the groups tend to merge indefinably into one another. In several instances strains possess characteristics which might place them in either of two groups. It is of interest that the strains which appear to approach one another most closely have in general been related to the same epidemic. This is particularly well illustrated by the strains in Groups *1* and *2*, all but one (Melbourne) of which were isolated during the epidemic of 1936–37. With the exception of the Gatenby strain those under heading *4* were obtained in 1935 and early 1936.

Although there appears to be a tendency for strains obtained in a given epidemic to group together, other evidence shows that different strains may be recovered from the same epidemic. The strains listed under *1* more closely approximate in their characteristics the strains of Group *2* than those of Group *4*, but the differences between the two groups are very definite. Differences between strains of the same epidemic are better illustrated by the Talmey and Gatenby strains, both of which were obtained in England not only during the same epidemic, but from the same district. The Talmey strain is much the same as the American strains listed under Group *1*, while the Gatenby strain belongs in a general way with the strains of Groups *4* and *4A*.

It is noteworthy that completely reciprocal cross-protection between strains does not necessarily hold. For instance, Gatenby serum completely protected against WS strain, and yet the WS antiserum afforded no protection against the Gatenby strain of virus.

It would seem a reasonable expectation that a strain of virus readily producing an antiserum of high titre for the homologous strain of virus would be more effective in eliciting heterologous antibodies than one which produces antiserum of low titre for the homologous strain. Such, however, is not necessarily the case. The Phila strain almost invariably produced antisera with poor protective capacities against itself, and yet the same antisera always exerted some protective capacity for mice against heterologous strains. Paradoxical though it may seem, antisera prepared against the Phila, Alaska and Gatenby strains repeatedly exhibited good protection against a heterologous strain of virus of standard strength, and yet afforded no protection for mice against the homologous strain. On the other hand, the WS strain produced

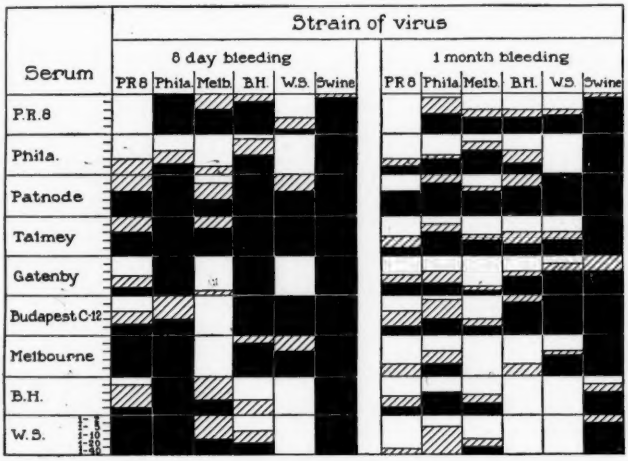

CHART II.—Comparison of serum of rabbits 8 and 30 days after vaccination.

sera of high titre against itself, although these sera failed to exert any protective capacity for mice against other strains.

Differences in zone of serological activity of serum obtained from rabbits at different intervals after inoculation.—It is important to emphasize that strain differences are most strikingly demonstrated when serum is obtained from rabbits early in the course of antibody response. After a longer period heterologous antibodies are more evident, and the differences somewhat more difficult to demonstrate.

This broadening of the band of serological response is well illustrated by an experiment in which sera taken from the same rabbits 8 days and again 1 month following a single inoculation of virus were tested in mice against strains of tissue culture virus. The PR8, Phila, WS, BH, Melbourne and swine strains of virus were tested against PR8, Phila, WS, BH, Melbourne, Talmey, Gatenby, Bud C12 and Patnode antisera.

All the sera obtained from the rabbits after 1 month possessed protective

antibodies for strains of virus against which the 8th day sera had failed to protect, and with all but two (Bud C12 and Patnode) some protective capacity for all strains of human origin was demonstrable. Moreover, Gatenby, BH and WS antisera obtained after 1 month showed some protective capacity against all strains, including the swine virus. The results are summarized in Chart II, in which the black, shaded and clear areas have the same significance as in Chart I.

In Vitro Neutralization Test.

The differential protective or neutralizing capacity of various antisera can be demonstrated *in vitro* as well as *in vivo*. In the following experiment a series of cultures was set up in 50 c.c. Erlenmeyer flasks, each containing 4·0 c.c. of Locke's solution and 5 drops of minced 13-day chick embryo. To a series

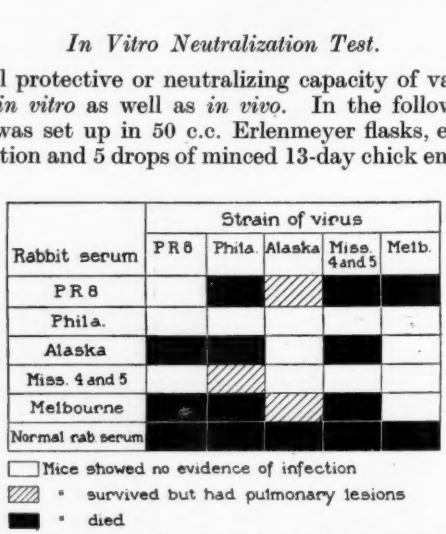

CHART III.—Results of neutralization tests in tissue culture.

of 5 flasks was added 0·5 c.c. of rabbit antiserum evoked against a single strain of epidemic influenza virus. Five such series, containing PR8, Phila, Alaska, Miss 4–5 and Melbourne serum, respectively, were prepared. To each flask of a sixth series was added 0·5 c.c. of normal rabbit serum. These 30 flasks were then arranged in 5 series of 6 flasks, each flask of the same series containing a different serum. To each flask of a series was then added 0·5 c.c. of a strain of culture virus, the PR8, Phila, Alaska, Miss 4–5 and Melbourne strains each being added to one series, respectively, so that each flask contained a different combination of serum and virus.

After 48 hours' incubation at 37° C., 0·5 c.c. of the supernatant from each flask was transferred to a fresh flask containing 4·5 c.c. of saline and 5 drops of minced 13-day chick embryo, but no serum. By this procedure the concentration of serum was again diluted 10-fold, so that the final dilution of serum was not less than 1 : 100—a point beyond which none of these sera protected mice against infection with full-strength tissue culture virus. Under these conditions virus which survived the original effect of serum should multiply without interference. The fresh cultures were then incubated at 37° C., and after 48 hours, 0·05 c.c. of supernatant from each was given intranasally to

groups of lightly anæsthetized mice. The mice were observed for 10 days, deaths were recorded, and on the 10th day surviving mice were autopsied and the degree of pulmonary involvement was noted.

The results are summarized in Chart III. Black areas indicate death, shaded areas survival with pulmonary involvement, and clear areas survival without pulmonary involvement. The results agree rather well with those obtained when the same strains of virus and the same sera were employed directly in mouse-protection tests. The PR8 antiserum completely neutralized the PR8 strain of virus and inhibited the growth of the Alaska strain, but had no effect on Phila, Miss 4–5 and Melbourne strains of virus. None of the strains from cultures containing Phila antiserum were infectious for mice, and of those in the Miss 4–5 serum only the Phila strain retained any demonstrable infectiousness for mice. The Alaska serum had no effect on PR8, Phila, or Miss 4–5 strains, but completely prevented growth of Alaska and Melbourne strains. The Melbourne serum prevented growth only of the Melbourne strain and partially inhibited the Alaska strain. Normal rabbit serum had no effect on any of the strains of virus.

Results as sharp as these must be significant. The fact that sera produced against strains of the same virus will to all intents destroy the homologous strain without apparent effect upon heterologous strains emphasizes the serological specificity of the respective strains.

Possible Alteration of Virus due to Prolonged Passage.

It has been suggested that certain strain differences are artificial and are due to one of two causes—prolonged animal passage or prolonged maintenance in tissue culture. The evidence obtained in this laboratory indicates that the strain differences are not related to prolonged animal passage. This was well demonstrated in an experiment in which 5 rabbits of the same breed, age and sex were inoculated with PR8 strain of mouse-passage virus—2 with the 285th passage and 3 with the 27th passage. The 27th passage was from material frozen and dried almost three years before, which was passed in mice several times to restore its virulence before being used for rabbit inoculations. The sera from all these rabbits gave essentially the same results when tested with strains of culture virus. Nor did the results differ in any significant manner from those previously obtained with PR8 antisera. Hence there was no demonstrable variation in the antigenic make-up of the strain in the period between its 27th and 285th mouse passages.

No change was detected in the antigenicity of the influenza virus during prolonged growth outside the animal body. A culture of the PR8 strain obtained from the 44th mouse passage and carried for 160 transfers in tissue culture was compared with a culture from the 70th mouse passage which had been transferred 130 times. Both gave identical results with the same sera in mouse-protection tests. Furthermore, protection tests performed with other strains of influenza virus after a few tissue culture transfers and again after 50 or 60 transfers gave strikingly similar results with the same sera which had been stored in the refrigerator in the interval between tests. After 380 transfers the PR8, Phila and swine strains have remained highly virulent for

mice and ferrets, while exhibiting no detectable change in their reactions toward various antisera. Other strains which have not been in tissue culture for so long a period as the PR8, Phila and swine strains also have remained, so far as can be detected, constant in their serological characteristics.

DISCUSSION.

It seems quite clear that the antigenic differences found to exist between strains of epidemic influenza virus are not due to alterations in the structure of the virus caused by either prolonged animal passage or by maintenance in tissue culture medium. The validity of the strain differences is emphasized by the work of Burnet (1937) and Stuart-Harris, Andrewes and Smith (1938), who, using less delicate methods, also found strains which varied in antigenic composition. The strain differences vary considerably in degree. Some are so slight that certain strains appear to be identical. On the other hand, some of the strains are so sharply different from the remainder of the strains of human origin as to suggest that at least one (BH) may be classified with the virus of swine influenza, and that the latter is merely a strain of epidemic influenza virus rather than a different but related virus.

Although the similarity of certain strains to one another makes it possible to arrange them in several groups, it seems inadvisable in the present state of knowledge to attempt too rigid a classification. Especially would it seem unwise to speak of types in the same sense as pneumococcus or streptococcus types, where there exists a very well-identified substance characterizing each type, and for which the respective antibodies are specific. In consideration of the variety of antibody response of rabbits to the various strains, it seems appropriate to compare the influenza virus with various bacteria showing a mosaic of somatic antigens, and not identified by a single specific surface antigen. With such a mosaic numerous strains might have common antigens, and yet, because of the variation in amount and arrangement of these, each strain would be antigenically different and possess its own peculiar characteristics. In this sense it is perhaps permissible to classify in " groups " or " types " those strains which possess similar antigenic patterns, no attempt being made to identify the strain by a single specific antigen.

Such a concept of the antigenic structure of the influenza virus renders less difficult the explanation of peculiarities in the antibody response of rabbits to various " groups " of strains. The virus particle of a strain such as WS, which evokes in rabbits a rather specific response, might well have a surface composed almost entirely of the dominant antigen, the interior or more inaccessible portions consisting of smaller amounts of other antigens. On the other hand, with the Phila strain in which the initial antibody response may be directed toward a heterologous rather than homologous strain, it is possible that the surface of the particle is composed of small amounts of different antigens, while the quantitatively greater or dominant antigen makes up the less accessible portions. Other strains may have structures with combinations between these two extremes.

Not only is it probable that the various strains of influenza virus vary in their antigenic patterns, but the marked differences in antibody response of

rabbits to Patnode strain (a weak strain which induces a good antibody response only for strains of its own group) and the BH strain (which not only is relatively unaffected by heterologous sera but evokes little antibody for heterologous strains) suggest the possibility that each strain may not contain all the antigenic components of all the other strains. The manner in which the characteristics of one group of strains tend to approach those of another group, although the outer boundaries are far apart, suggests that the various strains, or groups of strains, may be related to one another in much the same manner as the various colours are to the spectrum. The yellow, for instance, gradually fades on the one side through the orange toward the red, and on the other through the green toward the blue. The green and orange are related by a common factor, while the blue and red, each a factor in two related colours, are in themselves quite unrelated. Studies of the immunological relationships of the known strains of influenza virus have not as yet been thorough enough to permit a definite conclusion, but it does not seem impossible that there may exist strains which, while related to intermediate strains, are themselves antigenically unrelated.

Although the significance of the antigenic differences observed cannot be clearly evaluated at present, the findings presented in this report leave little doubt that the differences are of greater practical importance than was originally thought. For the identification of new strains of virus it seems quite likely that sera evoked against more than one distinct strain will be required. The strain differences are sufficiently marked to suggest that the epidemiology and immunology of epidemic influenza may be more complex than was considered to be the case on the basis of earlier virus studies. The readiness with which strains such as Patnode and Phila 37–7 are neutralized by heterologous sera, and the resistance of the BH and swine strains to the same sera, suggest that immunity to one strain may not necessarily confer protection against infection with another. This aspect of the problem will be considered in the following paper.

SUMMARY AND CONCLUSIONS.

Cross-neutralization tests performed with 24 strains of influenza virus are reported and the significance of the findings is discussed.

Although marked differences between some strains and striking similarities of other strains permit rough grouping, the characteristics of one group tend to merge into those of another. The strains which most closely resemble one another are, in general, those from the same epidemic of influenza, but serologically different strains may also be recovered from the same epidemic.

The specific effect of a given serum can be demonstrated by its action in tissue culture medium as well as by the usual mouse-protection test.

So far as it has been possible to determine, the antigenic differences between strains have not resulted from variations occurring in the course of animal passage or artificial cultivation.

It is suggested that the structure of the virus of epidemic influenza is a mosaic of antigens, numerous strains containing the same antigens arranged in such a way that each strain or group of strains is characterized by its own

peculiar antigenic pattern. It is further suggested that all strains may not contain all the antigenic components of all other strains.

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ANTIGENIC DIFFERENCES IN STRAINS OF EPIDEMIC INFLUENZA VIRUS: II. CROSS-IMMUNIZATION TESTS IN MICE.

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THE diversity of the antigenic constitution of different strains of epidemic influenza virus was demonstrated in the previous paper (Magill and Francis, 1938). The conclusion was reached that while many similarities exist between the various strains, there are also differences sufficiently great to permit the sharp identification of certain individual strains in the group. Moreover, additional differences of a lesser degree have been sufficient to indicate variations among member strains within the group, thus giving the impression that almost every strain has some serological property peculiar to itself.

It had been found in earlier experiments in ferrets, mice and swine that recovery from infection with epidemic influenza virus or swine influenza virus was followed by a state of active immunity to the other, although neutralizing antibodies to the heterologous virus were not ordinarily detectable in the serum of the resistant animal (Smith *et al.*, 1935; Shope, 1935, 1937; Shope and Francis, 1936). Moreover, repeated vaccination with one resulted in the production of circulating antibodies to both (Francis and Shope, 1936). Since cross-immunity could be obtained with these two viruses, which exhibit greater serological differences than any yet detected between strains of human origin, it was of interest to determine whether the serological differences demonstrated among the latter by passive protection experiments in mice were detectable

as well by experiments testing the ability of the various strains to induce active immunity in mice against other serologically different strains.

The present paper reports observations on the immunity induced in mice to intranasal infection with homologous and heterologous strains of epidemic influenza virus as a result of intraperitoneal vaccination with strains of different serological activities.

METHODS.

Virus.—Strains of influenza virus grown in tissue culture medium were used (Shope and Francis, 1936). Cultures, after incubation for 48 hours, were centrifuged free of tissue-cells and the supernatant fluid served as vaccinating material. The use of culture virus made easier the entire procedure of vaccinating and testing, since many strains of virus could be prepared for use at one time without worry as to the vagaries which inevitably attend mouse inoculation with strains of different virulence. Moreover, the greater number of strains approached a relatively uniform virulence for mice, although certain ones possessed consistently higher titres and others were consistently below the customary level. The titration of each strain of virus at the time of each vaccinating dose and at the time of infection was prohibited by the great number of animals which were required. Groups of mice were inoculated to test for the presence of virus, however, and some estimate of the virus content was obtained by recording the length of time intervening between inoculation and death. Titrations of all strains were conducted once or twice, at least, during the course of the experiments.

A total of 16 strains of epidemic influenza virus and 1 of swine influenza virus were used for cross-immunity tests. Twelve strains were obtained during the epidemic of 1936–37, the others during preceding epidemics. Their origins are detailed in the preceding paper.

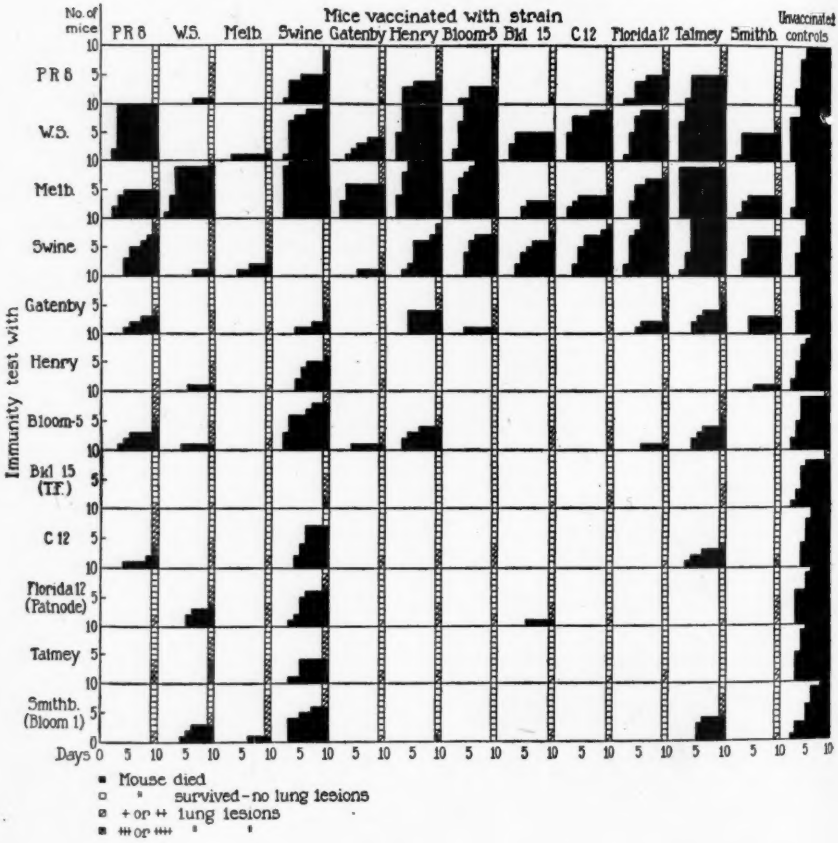
Vaccination and immunity test.—Large groups of Swiss mice of similar size and weight were used. Each animal was given two doses of 0·5 c.c. of culture supernatant fluid a week apart, and one week after the second dose groups of 10 mice each were tested for immunity by the intranasal inoculation of 0·05 c.c. of the various strains of tissue culture virus. It was found by preliminary tests that one vaccinating dose did not usually induce sufficient immunity to protect against the homologous virus, while it had been earlier established that repeated inoculations afforded a wide range of cross-protection. It was decided, therefore, to adopt the intermediate procedure mentioned. The mice were observed for 10 days after infection. All deaths occurring during this period were recorded, and at the end of 10 days the surviving mice were sacrificed and the extent of pulmonary involvement noted. In this manner it was possible to measure the efficacy of the immunizing procedure both in the prevention of fatal infection and in the more complete protection of the mouse against pulmonary injury.

Since it was obviously impracticable to conduct the entire series of vaccinations and infections at one time, with the majority of strains the vaccinations and the immunity tests against the various strains were carried out in two groups. Several groups of mice vaccinated against different strains of virus were usually tested at the same time, thus greatly reducing the number

of control animals required. The test dose of virus with the great majority of strains contained between 100 and 1000 lethal doses. In two instances 10,000 to 100,000 lethal doses were found, while in one or two cases 50 to 100 lethal doses were present.

RESULTS.

The Results of Cross-Immunity Tests.



The results of the cross-immunity tests in mice vaccinated with different strains of influenza virus are presented graphically in Chart I. The chart details observations on groups of 10 mice each for a period of 10 days. Horizontally each small block represents a day, while each vertical block represents a mouse. Thus the number of mice dead at a given time can be readily

ascertained. The characteristics of each strain can be studied both in its capacity to immunize mice against infection with other strains, and also in its capacity to overcome the immunity elicited in mice by other strains. It may be noted that pulmonary lesions present on the 10th day after infection are charted so that a partial state of immunity which does not prevent pulmonary infection is represented. A certain number of small lesions seem to occur in a great many mice tested with others than the homologous strain. Certain strains are designated in parentheses because their reactions were identical or nearly identical with the strain with which they are grouped. Furthermore, the size of the chart was thus reduced.

It is apparent that the immunity resulting from vaccination with any strain of virus was always effective in inducing complete immunity to that strain, but that mice so vaccinated showed varying degrees of immunity to other strains of virus. The greatest lack of cross-immunity was observed when mice vaccinated with heterologous strains were tested with PR8, Phila, WS and Melbourne strains and with swine influenza virus. These results are collected in the upper part of the chart, and represent to a great extent the failure of the strains obtained in the 1936–37 epidemic to immunize mice against infection with strains from human epidemics of 1933, 1934 and 1935 or against the swine influenza virus.

Immunization with swine influenza virus elicited a state of complete resistance to infection with the homologous virus, and, moreover, induced some degree of immunity against most of the strains of human origin. In the same manner immunization with epidemic influenza virus strains usually resulted in some degree of immunity to swine influenza virus.

In the lower right portion of the chart is seen the comparatively high degree of immunity which the strains of 1936–37 afforded against one another. Since the majority of these strains were of relatively equal virulence for mice the results of vaccination should be capable of direct comparison. Thus strains such as Henry, Bl 5, Fla 12 and Talmey, while behaving with considerable uniformity in their immunizing capacity against most heterologous strains, displayed certain differences in that vaccination with Henry strain failed to give complete immunity to Bl 5 strain. Furthermore, there was, in general, less active immunity to the Bl 5 than to the Henry strain. Similarly, the results with Talmey and Fla 12 strains differed only in that mice vaccinated with the former were less resistant to Bud C12 and Smithb strains than mice vaccinated with the latter. The Bud C12 strain appeared to be almost identical with the Bl 5. The Smithb strain presented certain characteristics which differentiated it from the preceding but related it closely to Bl 1 strain. The Gatenby strain differed somewhat from others of 1936–37 in that the latter did not appear to furnish as uniform immunity to it as to most strains in the group. Gatenby seems better related to the older strains.

Hence, on the basis of active immunity experiments, a tendency exists for strains of epidemic influenza virus obtained during the epidemic of 1936–37 to elicit cross-immunity within the group. Nevertheless differences did occur in the capacity of mice vaccinated with various strains in that group to withstand infection with any other given strain, thus indicating that complete homogeneity does not exist.

The PR8 and Phila strains of virus, while similar in virulence to most of the newer strains, appear to differ from them antigenically, as evidenced by the fact that an effective immunity against PR8 and Phila strains was not ordinarily induced by the 1936–37 strains.

It is interesting to note that the two human strains, WS and Melbourne, which gave the most uniform cross-immunity against heterologous strains, were also the ones against which all other strains exerted the least immunizing capacity. At the same time they were the two most virulent strains of tissue culture virus employed in this series of experiments. The possibility immediately arose that the apparent individuality of these strains was solely related to their greater virulence, and was quantitative rather than qualitative in nature. That this could not be entirely the case was shown, however, by the fact that while the two strains were of equal virulence, vaccination with the WS strain failed to immunize against the Melbourne strain, although immunity to the Melbourne afforded excellent cross-immunity to the WS strain. The two strains were, therefore, of obviously different antigenic constitutions.

Bkl 15 and TF strains which seemed practically identical were usually of somewhat lower virulence than the average, but nevertheless served as better immunizing agents than most of the other strains. This effect constitutes an example of still another difference almost impossible to control.

It seems a justifiable assumption, then, that the failure of strains of epidemic influenza virus to elicit in mice cross-immunity to other strains can be attributed to qualitative differences in the antigenic constitution of the respective strains. This assumption is particularly valid among strains of equal virulence, although some appear to give rise to a uniformly better immunity than others of somewhat greater virulence. It is quite striking that, in general, the results obtained in the present study of cross active immunity among strains of influenza virus conformed surprisingly well with the conclusions obtained in the preceding study with cross-neutralization tests. The strains tend to differ from one another, and also to group together in a manner similar to that observed with the serological tests.

Influence of Strain Virulence upon the Resultant Immunity.

Because of the suggestion that the results obtained with WS and Melbourne strains of virus were related to the greater virulence of these strains for mice, certain experiments were conducted to determine the effect of strain virulence upon the cross-immunity produced by strains of virus exhibiting the greatest differences in virulence.

Three groups of 50 mice each were given intraperitoneally 1, 2 and 3 doses, respectively, of 0·5 c.c. of fluid from a tissue culture of the Bkl 15 strain of epidemic influenza virus. The interval between successive doses was one week. The titre of virus in mice was such that deaths occurred in mice which received a dilution of 1 : 100 intranasally, and lesions were observed in mice receiving a dilution of 1 : 10,000 intranasally. Two weeks after the first

dose the mice which received only 1 or 2 vaccinations were infected intranasally. The other group of mice received its third vaccination at that time and was infected one week later. The groups of mice were divided into subgroups of 10 each and received the undiluted homologous Bkl 15 culture, undiluted, 1 : 100 and 1 : 10,000 dilutions of Melbourne strain of virus and undiluted swine influenza virus, respectively. The Melbourne strain of virus was of a virulence such that it caused death of normal mice in dilutions of 1 : 10,000 to 1 : 100,000 of tissue culture material. All survivors were sacrificed on the 10th day.

The results are presented in Table I. In this instance one vaccinating dose of the Bkl 15 virus was sufficient to induce a well-marked immunity to the homologous virus, and a partial immunity to the Melbourne strain in both the 1 : 100 and 1 : 10,000 dilutions. No demonstrable effect was observed against swine influenza virus nor against the full strength Melbourne culture. After two vaccinating doses there occurred a further enhancement of immunity to the homologous strain and to the diluted Melbourne culture and, in addition, a distinct resistance to the undiluted Melbourne material. No immunity to swine influenza virus was observed. With three vaccinations the immunity produced was apparently as effective against Melbourne strain of virus as against the homologous Bkl 15. The prevalence of small lesions in the lungs of all these groups of mice is unexplained.

Against swine influenza virus, the majority of animals receiving 3 doses of Bkl 15 culture developed sufficient immunity to escape fatal infection, but extensive pulmonary lesions were uniform. This is of interest, since the virulence of swine influenza virus culture was somewhat intermediate to that of the Bkl 15 and Melbourne strains.

Another experiment, with the WS and Melbourne strains, in which smaller numbers of mice were used, revealed that while the Melbourne, if anything, was somewhat more virulent than the WS strain, mice vaccinated with Bkl 15 culture developed immunity to the Melbourne strain, but not to the WS strain.

When a similar experiment was performed using the Melbourne strain for vaccinating purposes, almost complete immunity was produced against the homologous and Bkl 15 strains after one injection, but little effect was produced upon the swine virus. After the second dose the immunity to swine virus was equivalent to that produced by 3 doses of the Bkl 15 strain, but a third dose of the Melbourne culture resulted in no demonstrable addition to the immunity against swine virus. Moreover, when dilutions of the WS and Melbourne cultures were used for vaccination, so that the vaccinating dose contained approximately the same amount of virus as that used with 1936–37 strains, the immunity which resulted was not fully effective against 1936–37 strains, although completely protective against full-strength cultures of the homologous strains.

It appears, therefore, that quantitative factors play a role in creating an overlapping of immunity between strains. Further studies of the quantitative relationships will be reported elsewhere. If, however, with strains of epidemic influenza virus of relatively similar virulence, repeated doses of a strain of virus fail to induce immunity to another strain, the evidence seems to warrant the conclusion that the lack of cross-immunity is due to qualitative differences.

TABLE I.—*Effect of Repeated Vaccinating Doses on Resultant Cross-immunity.*

Immunity test with strain.	Mice vaccinated with Bkl 15 strain.										
	1 dose.						2 doses.				
Melbourne undiluted	3	3	3	3	4	.	5	5	6	+	+
	4	4	4	6	6	.	±	±	0	0	0
„ 1 : 100	4	4	5	6	+	.	+	+	+	±	0
	+	+	±	0	0	.	0	0	0	0	0
„ 1 : 10,000	6	7	8	++	++	.	0	0	0	0	0
	+	+	±	0	0	.	0	0	0	0	±
Swine influenza virus	4	4	4	4	5	.	4	4	4	5	5
	5	5	5	6	++++	.	6	7	7	7	+++
Bkl 15	++	+	+	0	0	.	0	0	0	0	0
	0	0	0	0	0	.	0	0	0	0	0

Immunity test with strain.	Mice vaccinated with Bkl 15 strain. 3 doses.						Unvaccinated controls.				
Melbourne undiluted	5	+	+	+	±	.	3	3	3	3	3
	0	0	0	0	0	.	3	3	4	4	4
„ 1 : 100	+	+	±	0	0	.	4	4	5	5	5
	0	0	0	0	0	.	6	6	6	6	7
„ 1 : 10,000	++	+	+	+	0	.	7	7	8	9	10
	0	0	0	0	0	.	10	10	++++	++++	++++
Swine influenza virus	6	6	+++	+++	+++	.	4	4	4	4	4
	+++	+++	++	++	+	.	4	5	5	6	7
Bkl 15	++	++	++	+	0	.	4	4	5	5	5
	0	0	0	0	0	.	6	7	7	7	8

Titration of Virus Strains.

Virus.	Virus dilutions.															
	10^{-1}			10^{-2}			10^{-3}			10^{-4}			10^{-5}		
Bkl 15	6	8	8	9	++++	+++	+++	++	++	+	+	0	0	0	0
Melbourne	3	4	5	4	4	5	4	6	6	6	8	8	+++	+++	++
Swine	7	8	9	9	9	++++	10	++++	+++	+++	++	++	+	0	+

Numerals represent day of death of individual mice.
± to ++++ = different degrees of pulmonary involvement at autopsy on the 10th day.
0 = no pulmonary involvement.

This is emphasized by the tests with swine virus, which in culture was of practically the same virulence as the majority of the epidemic human strains. In only one instance did a strain of human origin (Gatenby) produce a significantly good immunity against swine influenza virus. Even the more virulent strains failed to save the mice from extensive pulmonary injury. It is true, however, that immunity which results from vaccination with more virulent strains may develop more rapidly than is the case with strains of milder invasiveness for mice. On the other hand, the lack of reciprocal cross-immunity between the WS and Melbourne strains shows clearly that since both strains are of equally high virulence, the difference observed must necessarily be qualitative in character.

DISCUSSION.

The absence of complete cross-immunity to heterologous strains of epidemic influenza virus in mice vaccinated with a given strain tends to substantiate the significance of the serological differences between strains as shown by passive protection tests and reported in the preceding paper. It seems likely that the interpretation is in each instance the same: the differences represent variations in the antigenic make-up of different strains. The significance of variations in the distribution of group antigens in different strains is reflected by the individuality of the strain in creating a zone of cross-immunity which may not correspond completely with that elicited by another closely-related strain of the same virus. The tendency of 1936–37 strains to group together by serological tests is also demonstrable by cross-immunity tests. Minor differences between strains of the group are shown by both procedures, but more strikingly by neutralization tests. The manner in which the older strains differ from these strains and from one another is clearly brought out.

The lack of cross-immunity which has been described obtains only under the stated conditions of experiment. With minimal immunizing doses still further failure of reciprocal cross-immunity can be obtained, but there may also be failure to protect mice against pulmonary infection with the homologous strain. On the other hand, continued vaccination creates a state of complete cross-immunity between practically all strains of virus of human origin. The immunity developed as a result of vaccination with three doses of a strain of epidemic influenza virus seems to embrace not only the epidemic influenza strains, but is effective to a great extent in preventing fatal infections with swine influenza virus as well. In the latter instance, however, a much less complete cross-immunity is evident, for though the majority of mice survive infection with swine influenza virus, they usually exhibit extensive pulmonary lesions. This evidence again emphasizes the fact that all strains derived from human disease to date tend to comprise a group in which the individual strains bear a much closer relation to each other than any of them does to swine influenza virus.

Other quantitative factors apart from the number of doses of virus used for vaccination seem also to play a role in the extent to which cross-immunity can be demonstrated. While one vaccinating dose of the Bkl 15 strain of virus,

containing about 100 lethal doses of virus, failed to protect mice against infection with 10,000 lethal doses of the Melbourne strain, mice so treated exhibited distinct immunity to smaller doses. On the other hand, mice vaccinated with the Bkl 15 strain were more resistant to the Melbourne than to the equally virulent WS strain—clearly a qualitative effect.

One might minimize the variations observed in the capacity of various strains to induce a state of immunity which embraces all other strains of the same virus, were it not for the fact that immunity to epidemic influenza is generally thought to be of brief duration. Consequently a search for strain differences as an explanation of this phenomenon is not without practical connotation. Interestingly enough, the present observations tend to relate immunological differences in groups of strains or individual strains to different years and different epidemics. The importance to the problem of epidemiology of the differences herein described is not established by the present experiments. The cross-immunity which develops as a result of infection appears broader and more profound than that resulting from vaccination. Moreover, in the greater number of human individuals there appears to be a greater basic immunity than in mice and ferrets, so that the stimulation of infection probably produces an immunity reactive over a wide range of serologically different strains.

The significance of the results in the problem of preventive vaccination may, however, be more distinct. For while subcutaneous vaccination in ferrets modifies the disease produced by intranasal infection, it does not ordinarily prevent it entirely. The subcutaneous vaccination of mice does not produce as satisfactory an immunity as that which follows intraperitoneal inoculation. Consequently, in human individuals, when relatively small amounts of material are used for vaccination by the subcutaneous route, the inability of a strain to produce a broad state of immunity assumes greater importance. Since all strains tend to create an extensive zone of immunity when repeatedly inoculated, it may be that numerous inoculations of a potent strain will suffice for protection unless strains differing sharply from those heretofore isolated should be encountered. As an alternative, vaccination with a group of strains embracing the antigenic variations so far detected meets the theoretical needs.

It seems difficult on the basis of the present results to discuss with confidence the separation of epidemic influenza virus strains into types. Serologically, it is true, certain strains appear dominant, but, contrary to the opinion expressed by Burnet (1937), the impression has not here been gained that the strains of epidemic influenza differ so greatly from one another as does the human group from the swine group. The results obtained by immunity tests in only one direction do not justify the same conclusions as those derived from reciprocal cross-immunity tests, especially when a solution of the extremely difficult problem of controlling all factors of a quantitative nature has not been found. Nor is there sufficient evidence to eliminate the problem of spontaneous variation in strains as a result of the conditions of maintenance. It would appear for the moment, at least, that the term *type* should be reserved for future use when further study will have established the significance of current observations.

SUMMARY.

Cross-immunity tests were carried out with 16 strains of epidemic influenza virus and 1 of swine influenza virus in actively immunized mice. It was found that antigenic differences in strains such as demonstrated by serological tests in the preceding paper are reflected in a lack of cross-immunity. In general, strains obtained in the same year tend to be immunologically closely related, although individual differences exist. While quantitative factors are undoubtedly involved in the problem of cross-immunity between strains, qualitative differences can be shown in strains of equal virulence. The differences scarcely seem to warrant a designation of types, since most strains of human origin are closely related.

The importance of the serological and immunological strain differences to the problem of epidemiology and prophylaxis is discussed.

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SEROLOGICAL RACES OF INFLUENZA VIRUS.

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FOLLOWING the first isolation of human influenza virus (Smith, Andrewes and Laidlaw, 1933), several workers recovered strains from patients in different epidemics and in widely separated localities. It was assumed, chiefly on the basis of cross-immunity tests in ferrets, that all these human strains were immunologically identical, until Magill and Francis, in 1936, reported that two of their strains could be shown to be antigenically different by cross-neutralization tests in mice. This important discovery was confirmed both in Australia (Burnet, 1937) and in England (Andrewes, 1937 ; Stuart-Harris, Andrewes and Smith, 1938). Indeed, the wide dissimilarity found to exist between most of the strains isolated during the English epidemic of 1936-37 and our original strain WS, which was employed in a vaccination experiment on human volunteers, reported elsewhere (Smith, 1937 ; Stuart-Harris, Andrewes and

Smith, 1938) made it clear that improved methods of prophylaxis with more efficacious vaccines might emerge from an adequate analysis of the antigenic constitution of various strains of virus. An attempt to make such an analysis has now been completed with most of the strains available and, together with the application of the knowledge thus gained to a further study of prophylactic vaccination in ferrets and mice, forms the basis of the present report.

Cross-neutralization Tests in Mice.

Methods.

We carried out neutralization tests in mice by the methods previously described (Laidlaw, Smith, Andrewes and Dunkin, 1935). Filtrates of mouse lungs through gradocol membranes were mixed with falling five-fold dilutions of sera and, after about an hour's contact at room temperature, inoculated intranasally into anæsthetized mice. Mice were killed after 4 or 5 days and examined, the serum titre being taken as the final dilution which completely prevented the development of lung lesions. The sera chiefly used were obtained by bleeding out ferrets from the heart under chloroform anæsthesia 14 days after infection with one or other strain of virus. Our method differs from that used by Magill and Francis (1936) in three respects: firstly, we have worked entirely with mouse virus, not with virus propagated in tissue cultures; secondly, we have used the prevention of lung lesions, not death or survival, to determine our end-points; thirdly, we have used immune ferret instead of rabbit sera. Possible influences exerted by these factors in causing discrepancies between our work and that of Magill and Francis will be discussed later.

We had available for study 28 strains of virus, 17 coming from England, 1 from Russia, 1 from Hungary, 1 from Germany, and 8 from America. Sera capable of neutralizing each of the homologous strains to a high titre were obtained from convalescent ferrets as above described. The WS virus, isolated in 1933, was active in mice to a higher titre than any of the other strains, and filtrates of it were accordingly diluted, usually 1 in 10, with broth-saline before being compared with them.

Results.

Soon after we began to carry out cross-neutralizations between our various strains and sera, we found ourselves able to confirm Magill and Francis's finding that serological differences exist amongst human influenza viruses. In particular three strains, WS, Talmey and Gatenby, stood out as being sharply distinct from each other; later a fourth "specific" strain, Chr, was added to these. A table showing the results of a cross-neutralization test between 3 of the strains has been published previously (Stuart-Harris, Andrewes and Smith, 1938, p. 108). In this and similar experiments with these three strains, sera commonly had titres against the homologous virus of 1 in 250 or 1 in 1250, and against heterologous strains of 1 : 10 or 1 : 50.

As more and more strains were included in our studies it became increasingly difficult to perceive any order in the welter of cross-neutralization results. We accordingly decided to limit our efforts by studying the neutralization of all

strains only by (*a*) homologous sera, and by (*b*) those prepared against the four specific strains, and conversely the ability of all sera to neutralize those four viruses. The results were expressed in the form of diagrams (see Chart I). In previous studies we have expressed titres of sera in terms of a standard horse-serum ; this was not suitable for our present purpose because the virus used for its preparation was one of the most specific strains, and furthermore the horse was subjected to a long course of hyperimmunization. Consequently we took the homologous ferret-serum as the standard for each strain and compared heterologous sera against it, *e. g.* heterologous sera might be equal to S (= homologous serum), one-fifth as much (S/5) and so on. Each oblong or grid in the charts represents the antigenic analysis of a single virus. The top half of the grid indicates the neutralization of the strain in question by the four specific sera. Shading of a column up to the level of the top of the

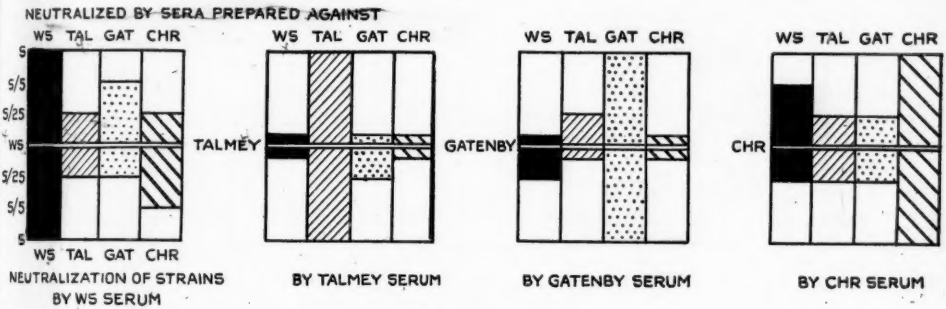

CHART I.—The four specific strains.

grid implies that the serum in question has a titre equal to that of the homologous serum ; shading up to $\frac{1}{3}$ or $\frac{2}{3}$ of the full height indicates serum titres of only S/25 or S/5. The scale is thus semi-logarithmic, but is otherwise similar to the diagrams used by F. W. Andrewes and Inman (1919) to illustrate their antigenic analysis of Flexner dysentery bacilli.

The lower half of the grid illustrating a particular virus (X) shows how the serum prepared against that virus (anti-X) neutralizes filtrates of the four specific strains. The results are expressed, much as before, as the titres of the anti-X serum in terms of the four homologous sera respectively. Thus in the WS diagram (lower half) the first column shows that WS serum neutralizes WS virus to full titre ; it naturally would, as it is itself the standard serum. From the second column it is seen to be less than one-twenty-fifth as effective against Talmey virus as is the standard anti-Talmey serum, and so on. In the ideal case the two halves of one grid would be mirror-images, and this is approximately so for the four specific strains ; poor inactivation of a strain X by a particular serum (anti-Y) is reflected in poor neutralization of Y by anti-X. The use of a serum of reference avoids errors due to variations in titre between different virus filtrates. Another source of error remains, however ; a particular ferret may yield an unusually potent serum, and the corresponding virus (Z)

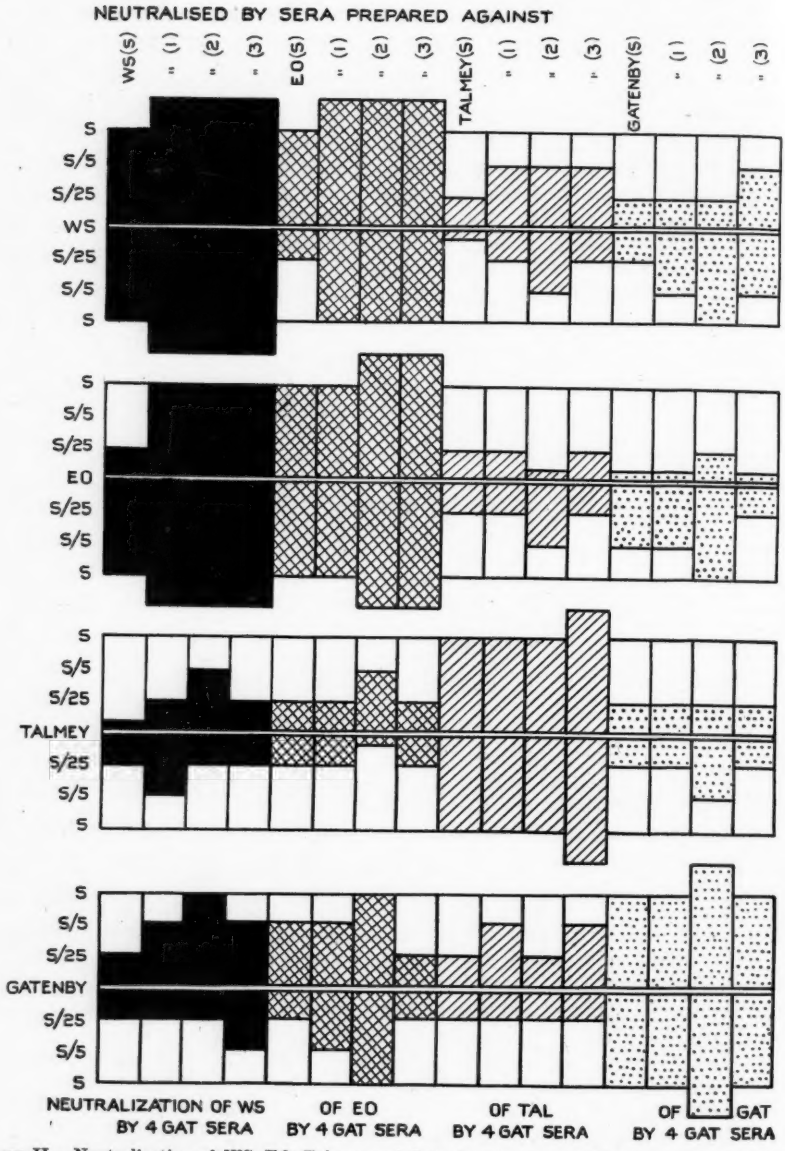

CHART II.—Neutralization of WS, EO, Talmey and Gatenby viruses by 4 sera prepared against each virus.

would then appear highly specific merely because the four standard sera were *relatively* ineffective. Herein lies some of the value of the lower halves of the grids. In the hypothetical case the anti-Z serum would be relatively active against the four specific strains and a diagram would result in which the columns above the central line would all be short, and those below it all long. Such an asymmetrical diagram should be regarded with caution; it would not indicate that a new specific strain had been found. A diagram with the opposite type of asymmetry (columns tall above, and short below the centre-line) would be met with if a particularly poor serum were encountered.

Repeated tests with the same viruses and sera gave concordant results so long as the sera were fairly fresh; where small discrepancies occurred, an average of the various results was used in constructing the diagrams. When several ferret sera were obtained against one virus and compared in cross-tests against other "specific" virus strains, again the results were reasonably concordant. Chart II shows the results of testing 16 sera against 4 viruses (WS, Talmey, Gatenby and EO), 4 sera corresponding to each virus being used. The EO strain was isolated by Dr. C. L. Oakley, of the Wellcome Physiological Research Laboratories, Beckenham, in 1937; for this virus and for most of the sera used in this particular experiment we are much indebted to Dr. Oakley. The chart shows that the EO and WS strains are practically indistinguishable, while the Talmey and Gatenby stand apart from them, and from each other. Except that one anti-WS serum neutralized EO virus poorly, sera all neutralized homologous virus (taking WS and EO as being identical), better than heterologous, the difference in favour of the homologous neutralization being, as a rule, twenty-five-fold, less often only five-fold.

Ferret sera were always inactivated for 30 minutes at 56° C. before use, as they otherwise may produce toxic lesions in mouse lungs; this inactivation did not appreciably affect their potency. Nevertheless, these inactivated sera gradually deteriorated on keeping in the cold for some months and were thus unsatisfactory as standards. The results we have charted have been obtained by the use of sera not more than 2 or 3 months old.

Chart III gives the antigenic analysis of 5 virus strains, 4 of which proved less specific than those we have hitherto discussed. Two, indeed, Bur and Kop, were extremely non-specific, being neutralized almost equally well by all four "specific" sera and producing sera which in turn neutralized all the four strains very well. BH virus proved to be another example of a strain almost identical with WS, while Mid and Bau were neither as specific as our specific strains nor as non-specific as Bur and Kop. Studies of 5 other European and 7 American strains were carried out at a time when our type sera had deteriorated somewhat; we do not feel sufficient confidence in the quantitative accuracy of our results with them to include their diagrams in the chart. Nevertheless it seems fairly certain that they belong in this "intermediate" group of viruses which are neither very non-specific nor very specific—a group which thus includes the majority of strains so far studied.

Four of our viruses isolated in 1937 readily infected ferrets, but failed to be adapted to mice. Though we had available, therefore, no virus in mice, we had sera corresponding to these strains, and we could test these against the specific strains. One of them (Oak) seemed to be closely related to WS,

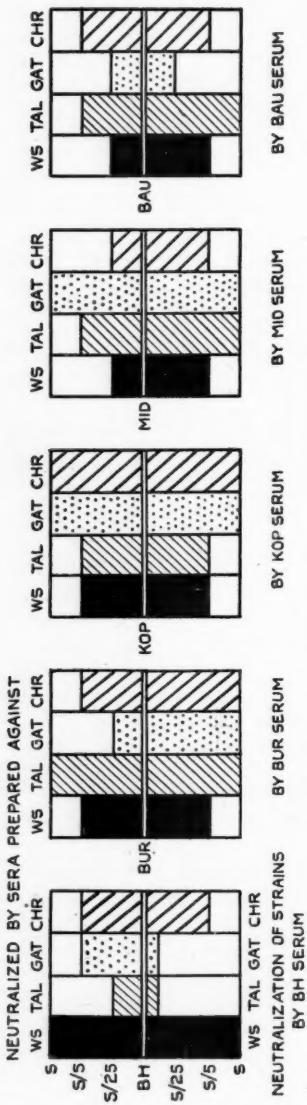

CHART III.—Analysis of five representative strains.

another (Cri) to be non-specific, and the others probably to belong to the intermediate group, but we do not feel any confidence in an analysis based on a study of serum only.

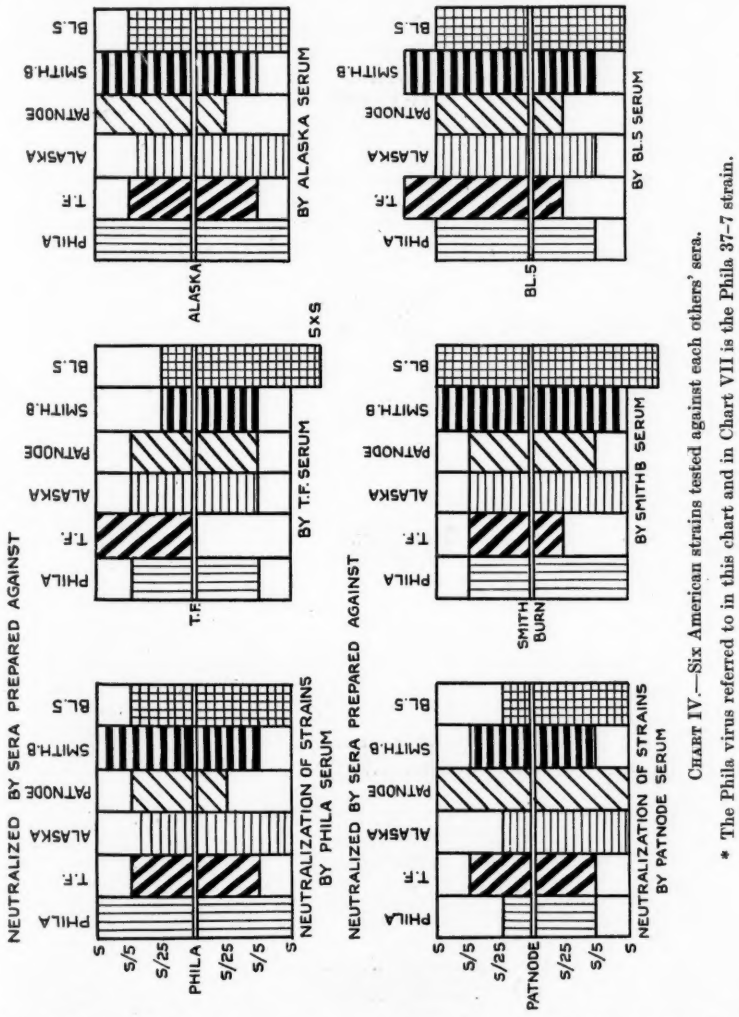

CHART IV.—Six American strains tested against each others' sera.

* The Phila virus referred to in this chart and in Chart VII is the Phila 37-7 strain.

Any falling off of the type sera would tend to make other strains under study appear to be more specific than they really were ; since none of the more recently analysed strains seemed to be very specific it is unlikely that we have wrongfully excluded any viruses from our specific group.

Further justification for not admitting as specific strains any of the American viruses came from a study of 6 of these kindly send us by Dr. Francis. We made ferret sera against these 6 strains, and put each virus up against each serum with the results shown in Chart IV. The strains evidently form a fairly homogeneous group, no strain standing out sharply as did our specific ones. The Patnode chart has the type of asymmetry associated with a particularly potent homologous serum (see above), and the Bl 5 chart an asymmetry of the other type, due to a very poor homologous serum.

In Table I are summarized our findings with various influenza strains. It has naturally been in some instances a matter of doubt as to how individual viruses should be classified, as differences are only quantitative.

Our results seem most easily interpreted on the assumption that 4 main antigens (? on the surface of the virus particles) are concerned in the neutralization test. Some strains contain predominantly one antigen and relatively little of the rest. In others on the other hand, all four antigens may be

TABLE I.—*Antigenic Analysis of Influenza Strains.*

Name of strain.	Source.	Results of antigenic analysis.	Remarks.
WS	London 1933	Specific strain WS type	
BH	„ 1935	„ „ „	
Sil	Moscow 1936	„ „ „	Less thoroughly studied
EO	London 1937	„ „ „	
Oak	„ 1937	„ „ „	Based on study of serum only
Chr	Shorncliffe 1937	Specific strain	
Talmey (Tal).	Chatham 1937	„ „	
Gatenby (Gat)	Windsor 1937	„ „	
Bur	Uxbridge 1937	Non-specific (master-) strain	
Kop	Berlin Dec. 1936	„ „ „	
Ham	London 1937	„ „ „	
Cri	Windsor 1937	„ „ „	Based on study of serum only
Mid	London 1937	Intermediate group most closely related to Gat strain	
Bau	Rugby 1937	Do. to Tal and Chr	
Ada	Shorncliffe 1937	„ Tal, Gat and Chr	
Bar	„ 1935	„ Chr	
Jor	Aldershot 1937	„ WS and Tal	Based on study of serum only
Mac	London 1937	„ Chr	
Viz	Hungary 1937	„ Tal	
RWF	Manchester 1937	„ Tal	
PR8	Porto Rico 1934	„ Tal and Chr	
Phila	Philadelphia 1935	„ Tal	Less thoroughly studied
Phila 37–7	„ 1937	„ Tal and Chr	
Alaska	Alaska 1936	„ Tal and Chr	
TF	New York 1937	„ Tal and Chr	
Patnode	„ 1937	„ Tal and Chr	
Smith B.	„ 1937	„ Chr	
Bl 5	„ 1937	„ Tal and Chr	

represented in fairly equal proportions, *e.g.* Bur; these may correspond to "master strains", such as have been encountered by Alexander (1938) amongst horse-sickness viruses. It is highly probable that evidence for the existence of other antigens could be found, but we feel that there are unlikely to be important antigens which have been overlooked, since all the strains studied were neutralized by one or more of our specific sera to a fairly good titre.

If this hypothesis of multiple antigens is correct, one might expect the top halves of our grid-diagrams to give more useful information than the bottom halves. We have sought for and failed to find convincing evidence of antigenic variations in our strains during propagation through ferrets and mice (*cf.* Stuart-Harris, Andrewes and Smith, 1938, pp. 109, 114); the susceptibility of a virus to neutralization by certain sera ought therefore to remain constant. On the other hand individual ferrets might well vary in their response to injections of a mixture of antigens, and different sera against one and the same strain might accordingly differ in their behaviour when tested against a number of strains. We obtained some evidence that such differences do in fact occur, particularly with sera corresponding to viruses of the intermediate group.

Comparison with American Results.

Magill and Francis (1936) found by the aid of immune rabbit sera sharp serological differences between two virus strains—PR8 and Phila. By our tests these and all the other American strains we have had fall into the intermediate group, and are not sharply distinguishable from one another; only minor differences are recognizable, such as occur between any two strains in this group. Burnet also (1937) finds by neutralization tests on egg-membranes that the PR8 and Phila viruses are closely related to each other and distinct from WS. When we first encountered this discrepancy between the American results and our own, we thought it possible that the cultivation of strains in tissue-culture in America might have rendered them more specific (Andrewes, 1937); further study has not supported this suggestion. Dr. Francis informed us by letter that he could only demonstrate the differences in question with rabbit, and not with ferret sera. We prepared immune rabbit sera according to the technique described by Magill and Francis and readily obtained active sera. These were, as expected, much less potent than sera from recovered ferrets, neutralizing homologous filtrates only at dilutions of about 1 in 50, and filtrates of unrelated strains at 1 in 10, 1 in 2, or not at all. There was, however, no difference in specificity between ferret and rabbit sera; our rabbit sera failed, as our ferret sera had done, to show up clear-cut differences between the Phila and PR8 viruses.

Table II shows a neutralization test between these two viruses, rabbit sera being used.

TABLE II.—*Cross-neutralization Test with Rabbit Sera.*

	Phila virus.	PR8 virus.
Anti-Phila serum . . .	50 .	50
Anti-PR8 serum . . .	50 .	250

(The figures give the highest serum dilution which completely neutralized a virus filtrate.)

We next tested the possibility that rabbit sera would distinguish between these two strains if we took survival of mice instead of prevention of lung lesions as our end-point. Once more, however, no important difference between the strains was demonstrable. It may be mentioned at this point that the death-or-survival titration method was not applicable to many of our strains, as they were not sufficiently virulent to kill mice with regularity.

The cause of the divergent results on the two sides of the Atlantic remains, therefore, obscure. It seems likely that Francis and Magill are studying differences amongst strains of our "intermediate" group, differences which we tend to neglect as being much less striking than others we have encountered.

Antibody-absorption Experiments.

Attempts were made to throw light on the antigenic structure of influenza viruses by absorption of neutralizing antibodies. After a number of trials the following technique was adopted for the absorption test: a dilution of serum was chosen which could be relied upon to inactivate 100 m.i.d. of virus. This was mixed with a fresh unfiltered 5 per cent. suspension of infected mouse lungs, and absorption was carried out for 2 hours at 37° C. and overnight in the refrigerator. The excess virus used for absorption was then removed by filtration through a gradocol membrane which would retain the virus (a.p.d. 0·1μ) or, more conveniently, by heating for 30 minutes at 56° C. The absorbed serum was then tested for neutralizing properties against dilute virus filtrate (about 100 m.i.d.) and compared with a serum which was unabsorbed, but otherwise subjected to the same procedures. For this neutralization test virus and serum were incubated together for 2 hours at 37° C. before being tested intranasally on etherized mice. We have shown previously that such incubation is desirable when dilute reagents are being employed (Andrewes, Laidlaw and Smith, 1935). Control experiments showed that no absorption was effected by suspensions of normal mouse lungs. A protocol of an absorption test is given in Table III.

TABLE III.—*Protocol of Antibody Absorption Test.*

		WS ferret-serum		
			Absorbed by	
Final serum dilution.	*Nil* (broth saline control).	Unabsorbed.	Undiluted virus.	Virus 1 in 10.	Virus 1 in 100.
1 : 500	2, 3, 2	0, 0, 0	1, 2, 2	2, 2, 3	0, 0, 0
1 : 2500	—	0, 0, 0	3, 2, 2	1, 2, 3	2, 1, 3

The figures show the extent of lung lesions, ranging from 0 (normal lungs), 1 (1–3 mm. lesions) to 3 (extensive lesions).

Sera were tested in duplicate against WS filtrates in final dilutions of 1 : 10^4 and 1 : 10^5; essentially the same results were obtained in each instance.

In the example shown in the protocol, absorption of antibody from dilute serum has been obtained with undiluted virus, virus 1 in 10 and, in the case of the highest serum dilution only, by virus 1 in 100.

We have readily obtained such results when using the very potent WS virus and homologous horse, ferret or rabbit sera. Absorption has also been effected by WS virus filtrates or by unfiltered virus inactivated by heat or

formaldehyde. With other strains we have, however, been less successful. We have failed with strong suspensions of swine influenza virus in the form of mouse lung suspensions to absorb the antibody from a homologous horse-serum; nor could we, except in a single experiment with Talmey virus, absorb any antibodies from their corresponding rabbit or ferret sera with Talmey, Phila or Bur viruses. Probably these failures were largely due to the fact that these viruses were all active to lower titres than the WS virus, and our absorbing suspensions consequently contained many fewer virus particles. We did, however, succeed in absorbing with WS virus suspensions the antibodies active against WS contained in anti-Phila rabbit-serum, and anti-Talmey and anti-Bur ferret sera. These absorptions with WS virus showed that absorption of a serum with a foreign strain can remove the antibodies corresponding to that strain while leaving others untouched. This is what would be expected by analogy with experiments with anti-bacterial sera.

Burnet and Lush (1938) have produced evidence that different anti-influenzal sera differ in the readiness with which their antibodies are absorbed; they suggest that the antibodies are of varying avidity. Such differences in the avidity of sera may very well have affected the results of our absorption experiments.

Cross-immunity Tests in Mice.

In a previous publication (Stuart-Harris, Andrewes and Smith, 1938) it was stated that the antigenic differences existing between virus strains and demonstrable by the serum neutralization test in mice might well be a determining factor in the outcome of attempts at prophylaxis. However, our attempt to correlate the levels of human and swine strain serum antibodies in vaccinated mice with the degree of their active immunity against each strain, together with the earlier observations of Francis and Shope (1936) on cross-immunity in recovered animals, emphasized the need for caution in arguing from one type of experiment to another. Indeed it was expressly stated in our previous report (p. 135) that "protection against a heterologous virus strain may be afforded, although the serological response, as judged by our present methods of assay, would appear to be strictly homologous". It was thus obvious that active immunization experiments with different strains were required in order to obtain essential information as to the practical importance of these antigenic dissimilarities. One such experiment with WS and Talmey strains was reported in the publication cited, and appeared to show that the protection induced by vaccination with either strain was much more effective against the homologous virus, although there was also a considerable degree of cross protection against the heterologous strain.

Methods.

Groups of mice (usually 12 per group) were given 2 doses of the various vaccine preparations to be tested. The first dose of 0·2 c.c. was given subcutaneously, the second of 0·5 c.c. intraperitoneally a fortnight later. Untreated control groups were always kept under strictly comparable conditions. Two weeks after the second immunizing inoculation, both treated and untreated

groups were given a test dose of virus by the routine method of intranasal instillation under ether narcosis. Deaths were recorded for 14 days, after which all survivors were killed and their lungs carefully examined for the presence of specific influenza lesions. The variations in the numbers in various groups shown in the charts are due to occasional non-specific deaths during the six weeks period of the experiment, and the loss of occasional mice during the test inoculation under anæsthesia.

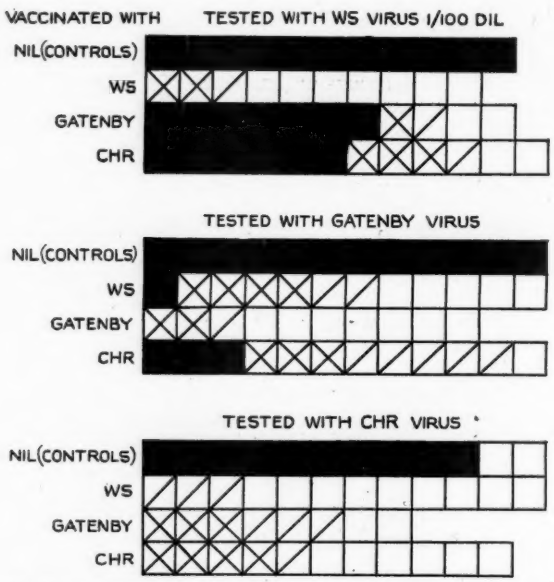

CHART V.—Cross-immunization experiment with three specific strains of virus.

In this and subsequent charts each horizontal column represents the outcome of the immunity test on a group of mice immunized as indicated.

■ = Mouse died of influenza within 14 days.

⊠ = Mouse with extensive lung lesions when killed after 14 days.

⊘ = „ „ small „ „ „ „ „ „

□ = „ „ no „ „ „ „ „ „

Results.

Experiment 1 (Chart V) was a cross-immunity experiment with three of our specific strains. The best protection against each of the viruses was conferred by vaccination with the homologous strain except in the case of infection with Chr virus; here the group vaccinated with WS virus enjoyed better protection than the group vaccinated with homologous Chr virus. It should be noted,

however, that the WS filtrates used as vaccines had a hundred-fold higher titre than either the Gatenby or Chr vaccines. The influence of amount of virus injected upon the prophylactic efficacy of vaccination has been reported previously (Andrewes and Smith, 1937). Nevertheless, a similar difference of titre between WS and Gatenby vaccines did not prevent Gatenby from exerting a greater effect than WS against Gatenby virus infection. Reference to Chart I, however, will show that WS and Chr have more antigen in common than have WS and Gatenby. It is, of course, possible that the anomaly was caused by different amounts of dead virus in the various vaccines.

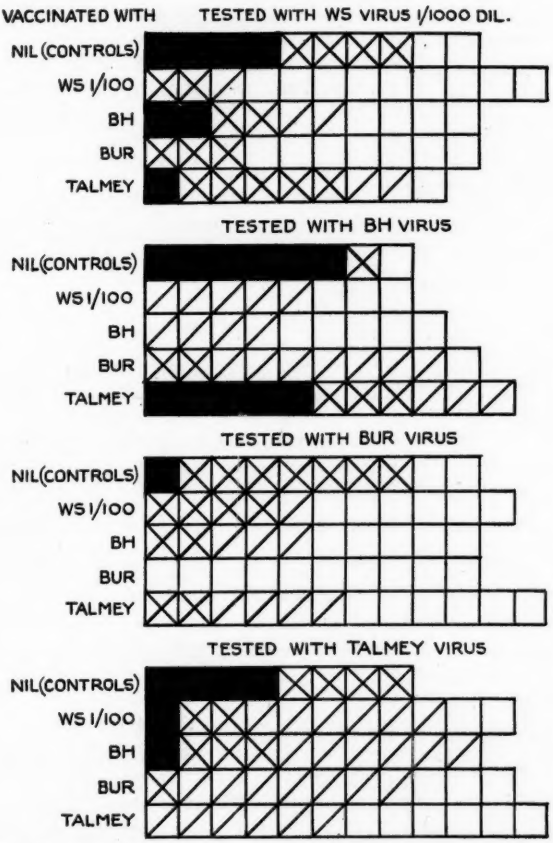

CHART VI.—Cross-immunization experiment with specific and non-specific virus strains. See legend at foot of Chart V.

Experiment 2 (Chart VI).—The experiment was designed to compare the immunizing values of two specific strains belonging to the same type (WS and

BH), a specific strain of different type (Talmey), and a non-specific or " master " strain (Bur) which contains large amounts of the specific antigens dominant in both WS and Talmey. The WS and BH strains are very closely related, and, apart from a slight advantage in favour of WS vaccine against WS infection, there would appear to be no significant differences between them as regards prophylactic efficacy. The most important feature of the results is the superiority of (Bur), the non-specific strain, over (Talmey), the specific strain, when protection is sought against heterologous infection. The suggestion is that if only one strain were available as a vaccine, a non-specific one would be preferable to a specific one.

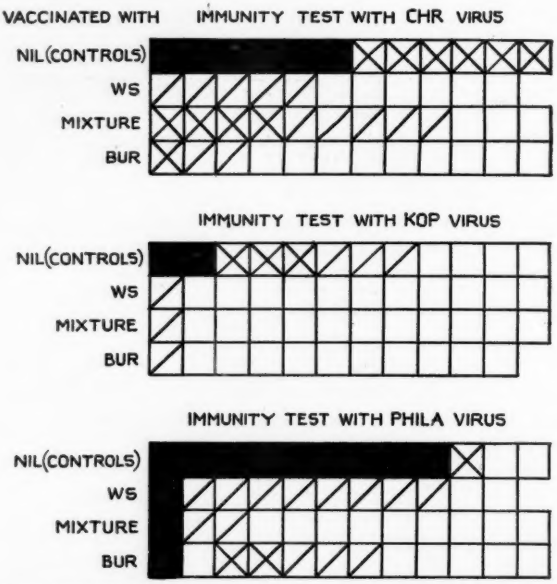

CHART VII.—Cross-immunization experiment with specific, non-specific and mixed vaccines. See legend at foot of Chart V.

Experiment 3 (Chart VII).—In this experiment the three vaccines employed were a specific virus (WS), a non-specific virus (Bur) and a mixture of three specific viruses of different type (WS, Talmey, Gatenby). The immunity tests were not made with the strains used for immunization, but with other strains representing the various antigenic types, Chr (specific), Kop (non-specific), and Phila 37–7 (intermediate). The experience of the 1936–37 outbreak had shown that an epidemic may be caused by not one, but several different virus strains, and it was hoped that the result of the experiment might indicate the best sort of vaccine for conferring protection against exposure to many strains, not themselves available for incorporation in the vaccine. It will be seen

from the chart, however, that none of the vaccines was consistently better than the others. The Kop test virus was relatively so weak that only two of the untreated control mice died and four of them escaped infection altogether ; it is therefore not very surprising that all the vaccines gave practically complete protection against Kop infection. With the other test viruses, Bur vaccine gave the best protection against Chr, and mixed vaccine best against Phila 37–7. A feature of the results is that whereas mixed vaccine gave better protection against Phila infection than did WS vaccine, the reverse was true against Chr infection ; that is, the presence of strains additional to WS in a vaccine was advantageous when one test virus was used, but disadvantageous with another test virus.

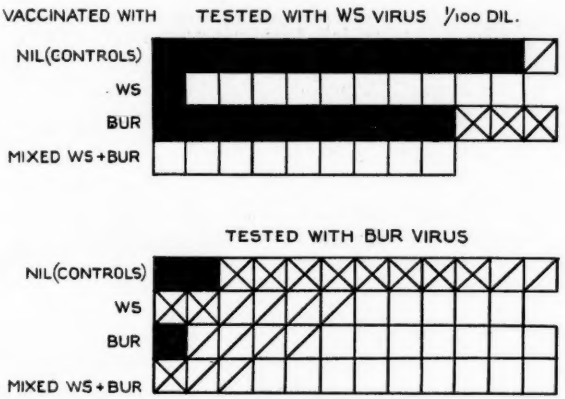

CHART VIII.—Cross-immunization with single strain and mixed vaccines. See legend at foot of Chart V.

Experiment 4 (Chart VIII).—The effect upon the efficacy of vaccines of adding an additional strain was directly investigated. WS, Bur and mixed WS plus Bur vaccines were used, and the immunity tests were made with the same two strains. The single strain vaccines each consisted of virus filtrate diluted with an equal volume of saline ; the mixed vaccine was a mixture of equal parts of the two filtrates. It is obvious, therefore, that unless one strain in a mixture interferes in some way with the immunizing activity of another, the effect of the mixed vaccine ought to be a summation of the protection induced by the homologous single vaccine and the cross protection induced by the heterologous vaccine. The chart shows that this, in fact, was what occurred ; the mixed vaccine was superior to the homologous single vaccine with each of the test viruses. It is important to bear in mind, however, that the mixed vaccine contained homologous virus for each of the tests. In cases where the infecting strains were different from all strains incorporated in a mixed vaccine, the decisive factor might well be the relative amounts in the vaccine of the various antigenic components contained in the infecting virus.

Cross-immunity Tests in Convalescent Ferrets.

Cross-immunity tests in ferrets often fail to show the antigenic dissimilarities amongst various strains of virus which are revealed by cross-neutralization tests in mice. Ferrets which have recently recovered from infection with one strain are usually, but not always, solidly immune to infection with a heterologous strain. Thus of 18 ferrets convalescent from WS virus infection, only 2 proved susceptible to infection with some other human strain ; conversely, of 12 ferrets convalescent from infection with a strain other than WS, only one was susceptible to WS virus. Yet the WS strain has been shown by the cross-neutralization tests in mice to be one of the most specific of the human strains so far isolated. Indeed it has been previously reported that similar cross-immunity occurs in ferrets which have recovered from infections with such widely different strains as human (WS) virus and the swine influenza virus of Shope (Smith, Andrewes and Laidlaw, 1935).

Duration of Heterologous Immunity.

This heterologous immunity may well be the determining factor in the protection of an individual throughout the exposure to risk period of an epidemic, particularly if the epidemic embraces several antigenically different virus strains as did the outbreak of 1936–37 ; its duration therefore is a matter of considerable importance.

Six ferrets were inoculated intranasally with Gatenby virus under ether narcosis ; each suffered an attack of influenza with characteristic temperature response and nasal symptoms, followed by complete recovery. At intervals of 3 weeks, 6 weeks and 3 months after the primary inoculation, pairs of ferrets were bled by cardiac puncture, and immediately thereafter were subjected to an immunity test with WS virus. The serum samples were subsequently titrated against WS virus by the mouse-neutralization test. The two ferrets tested three weeks after infection proved completely immune, of those tested at 6 weeks, one was immune, and the other susceptible, whilst both animals tested at 3 months were susceptible. The three susceptible ferrets had typical attacks of influenza so far as temperature and symptoms were concerned, and at autopsy their turbinates showed congestion and necrosis with muco-pus in the nasal sinuses ; none of them, however, had any lung involvement, although a control ferret inoculated with the test virus showed lung lesions on each occasion. In each pair the serum of one ferret was estimated to possess WS antibodies equal to standard, that of the other equal to S/25 to S/5. The apparently high values are considered to be due to the fact that the sera were titrated in parallel with a WS standard serum which had been kept for months, and was known to have declined in potency. There was evidently not a close correlation between level of heterologous serum antibodies and active heterologous immunity. The results suggest that heterologous immunity tends to fade sooner than the resistance against homologous virus, for it has been shown previously that homologous immunity usually remains solid for about 3 months following a primary infection.

Vaccination of Ferrets against Gatenby Virus.

All our previous attempts to immunize ferrets by vaccination were made with either the WS or swine strains of virus. Both these strains have attained greatly enhanced virulence during the course of our work, and it was thought that this might account for the consistent failure to confer solid immunity by vaccination, especially in view of the fact that sufficient resistance for the withstanding of contact infection is easily conferred (Smith, 1937 ; Stuart-Harris, Andrewes and Smith, 1938, p. 125). An experiment with a fairly recently-isolated strain (Gatenby) was therefore made.

Three ferrets were given either two or three subcutaneous doses of living Gatenby mouse virus at fortnightly intervals. Two weeks after the last immunizing dose they were tested along with two normal controls by the intranasal instillation of Gatenby virus under ether narcosis. In spite of the fact that it was a recently-isolated strain the virus caused patches of lung consolidation in both control ferrets as well as the characteristic temperature response and nasal symptoms. The vaccinated animals presented a striking contrast, although none of them could be regarded as completely and unequivocally immune. In two the temperature just touched 104° on a single occasion, the other remained below this level throughout. All three were recorded as having moist noses, but at autopsy their turbinates and nasal sinuses showed at most a slight moistness. The lungs of all three remained entirely free from influenzal lesions. The vaccination, therefore, resulted in almost complete protection even though the strain employed was capable of attacking the lungs.

Vaccination of Ferrets with Basic Immunity.

Previous studies have shown that the vaccination of ferrets possessing basic immunity as a result of a past attack of influenza usually confers full protection against infection with the homologous strain of virus. This reinforcement of active immunity is accompanied by a striking increase of the homologous serum antibodies (Smith, Andrewes and Laidlaw, 1935 ; Stuart-Harris, Andrewes and Smith, 1938, p. 128).

Unfortunately the multiplicity of virus strains may make homologous immunity of less moment than resistance against heterologous strain. We therefore studied the effect of vaccination upon heterologous immunity by experiments with ferrets already possessed of some degree of resistance, such as we believe the majority of human beings enjoy.

Experiment 1 (Table IV).—The two virus strains WS and Phila 37–7 were employed both as vaccines, and for the subsequent immunity tests. Eighteen ferrets, each of which had passed through a typical attack of influenza from 8 to 15 months previously, were divided into six groups of three, the groups being made as nearly as possible comparable with respect to the past influenzal histories. Two groups were left untreated to serve as controls ; the others were vaccinated, 2 with WS virus, 2 with Phila 37–7 virus. The vaccines used consisted of 5 per cent. suspensions of infected ferret's lungs, and the dose, in all cases a single dose of 5 c.c., was given subcutaneously. All the animals received an immunity test under ether anæsthesia 2 weeks later, with either

WS or Phila 37–7 virus, as shown in Table IV. Two normal control ferrets were also inoculated with each virus strain so that the amount of basic immunity possessed by the unvaccinated convalescent ferrets might be estimated. All the ferrets were killed on the 5th day following the immunity test.

TABLE IV.—*Vaccination of Ferrets with Basic Immunity followed by Cross-immunity Tests.*

Ferret number.	Previous history.	Vaccinated with.	Tested with.	Result of test.		Lesions at autopsy in	
				Symptoms.	Fever.	Turbinates.	Lungs.
49/38	Normal control	..	WS	++	++	+++	++
54/38	„ „	..	„	++	±	+++	+++
140/37	WS +	..	„	+	+	+	0
29/37	Har + WS +	..	„	+	++	+	0
168/36	Pea + WS −	..	„	+	++	++	0
10/37	Ord + WS −	WS	„	0	0	0	0
138/36	WS + Mid +	„	„	0	0	0	0
101/37	Bur + WS −	„	„	0	0	0	0
175/36	Kop + WS −	Phila 37–7.	„	0	0	0	0
95/37	WS +	„	„	0	0	0	0
168/37	WS +	„	„	0	0	0	0
51/38	Normal control	..	Phila 37–7.	++	++	+++	++
55/38	„ „	..	„	++	++	++	+
193/37	WS +	..	„	+	++	++	++
92/37	Tal + WS −	..	„	+	++	+	0
187/36	Nix + WS −	..	„	+	++	++	0
143/37	Dip + WS −	WS	„	+	+	+	0
169/36	Cri + WS −	„	„	0	0	0	0
50/37	Ha + WS −	„	„	0	0	0	0
167/36	Ste + WS −	Phila 37–7.	„	0	0	0	0
137/37	WS +	„	„	+	+	±	0
14/37	WS +	„	„	0	0	0	0

The results are summarized in Table IV. In the column headed " Previous history ", we have indicated the strain of virus responsible for the primary attack of influenza, and in cases where a subsequent cross-immunity test was made, the result of such test. A comparison of the normal controls with the unvaccinated convalescent controls shows that the latter had sufficient basic immunity to protect the lungs in every case except one (F193/37). This basic immunity was also evidenced by the relatively milder symptoms and slighter degree of turbinate involvement in the majority of cases. It is practically certain that the groups of vaccinated ferrets must have possessed similar basic immunity prior to their vaccination, so that, had they been left untreated, they too would have responded to the immunity test with a mild attack of influenza without any lung involvement. The vaccination, however, converted this partial immunity into solid immunity in all cases except ferrets 143 and 137, which suffered exceptionally slight attacks of illness as a result of the final immunity tests with Phila 37–7 virus. F143 had been vaccinated with heterologous WS virus, F137 with homologous Phila 37–7 virus. The results indicate that it may not be necessary to use the virus strain which

caused the primary attack of influenza in order to stimulate the residual immunity; furthermore it seems likely that the immunity thus reinforced may prove effective against yet a third strain.

TABLE V.—*Vaccination of Ferrets with Basic Immunity.*

Ferret No.	Vaccinated with.	Tested with.	Result of immunity test.
117/36	..	Phila 37–7.	Typical attack of influenza.
66/37	..	„	„ „ „
83/36	WS	„	Very mild attack.
131/36	„	„	Mild symptoms, without fever.
36/37	Mixed strains	„	„ „ „ „
92/36	„	„	„ „ „ „
84/36	Bur	„	„ „ temperature 104·4 on one occasion.
154/36	„	„	„ „ temperature rose on 4th day only.
251/37	Normal control	„	Severe symptoms and turbinate involvement. No fever.
252/37	„	„	Very severe attack with pulmonary involvement.
95/36	..	WS	Typical attack.
55/36	..	„	„ „
67/37	WS	„	Immune.
96/36	„	„	Mild symptoms, without fever.
113/36	Mixed strains	„	„ attack.
43/36	„	„	„ symptoms, without fever.
130/36	Bur	„	Very mild attack.
72/36	„	„	Mild symptoms, without fever.
253/37	Normal control	„	Very severe illness, with death from influenzal pneumonia.

Experiment 2 (Table V).—A similar experiment was made in which three different vaccines were used; namely, a specific virus (WS), a non-specific "master" strain (Bur) and a mixture of our four specific strains, WS, Gatenby, Talmey and Chr. Instead of infected ferret lung-tissue, which was not available for all these strains, 5 per cent. suspensions of mouse lungs were employed. Dosage and time intervals were the same as in Experiment 1. Final immunity tests were made with WS and Phila 37–7 strains, both of which usually cause extensive lung involvement if inoculated under ether anæsthesia. The system of recording results utilized in Table IV would be of little value in this experiment, because only one animal (F67/37) remained absolutely free from all symptoms of infection. All the vaccinated ferrets, however, withstood this immunity test much better than the controls; instead of typical attacks of influenza they had extremely mild attacks, in many cases afebrile, in which symptoms like "wet nose" and "slight nasal obstruction" occurred only at irregular intervals. The autopsy findings also emphasized this difference, for the nasal passages of vaccinated ferrets were either normal or showed very slight changes compared with the frank congestion and muco-purulent exudate found in the unvaccinated. It is of interest that, apart from the particularly good homologous immunity induced by the WS vaccine, all three vaccines were equally efficient in stimulating resistance against either of the test strains.

DISCUSSION.

The work presented here establishes beyond reasonable doubt that very many strains of influenza exist, which differ in their antigenic constitution. Such differences are most clearly revealed by cross-neutralization tests in mice, but the active immunization experiments with mice indicate that the dissimilarities are not merely of academic interest, but may have great practical importance. It is probable that they are of significance in the many epidemiological puzzles presented by epidemic influenza. Doubtless, too, the second attacks suffered by some patients too long after the first illness to be considered merely a relapse, but too soon to fit in with what we know of the usual immunological response, may be explicable on the basis of exposure to antigenically dissimilar strains of virus. Whether the divers strains are fixed in antigenic constitution or vary from time to time, especially during epidemics, by the sporting of mutant forms we do not know. Although speculations upon their genesis are interesting, we propose to limit discussion here to their significance in the major problem of prophylactic immunization.

It seemed to us that for the solution of this problem it was essential to make an antigenic analysis of the numerous strains at our disposal, to see whether any sort of system and order could be found in the apparently hopeless confusion. It is as yet impossible to present any rigid classification, but enough has been done to show that probably all human strains (and the swine strain also) possess some antigenic factors in common, so that a low grade of non-specific or " group " immunity follows infection with any strain, and is effective to some extent in reducing the chances of reinfection with any other strain. The enhancement of this " group " immunity to as high a level as possible may possibly be the most effective method of prophylaxis. Other antigenic factors confer specificity ; and of these there are not a few. It may even be true that no two strains are identical, but have minor differences dependent upon the quantitative distribution of antigens, if not upon qualitative differences. However that may be, it has been possible so far to delimit only certain broad categories amongst the strains available for study ; these we have called the specific, of which we have so far recognized four distinct types, the non-specific or " master ", and the intermediate strains. From theoretical considerations one would expect that the immunological response to infection or artificial immunization with a specific strain would be primarily concerned with the specific or dominant component and that later it would broaden to embrace the various other components. This expectation is borne out in practice. Highly specific sera can only be obtained early in convalescence, or shortly after vaccination, whilst the long-continued hyper-immunization of a horse in order to obtain a standard serum resulted in a product with an exceptionally wide range of activity.

The problem of choosing the best vaccine for active immunization is therefore difficult. The experience of the last epidemic taught us that even in a single outbreak of influenza widely dissimilar virus strains might be encountered. It is unlikely that any virus vaccine will be equally effective against each and every strain, so that the complete protection of every vaccinated

individual throughout a big epidemic cannot be expected. From the experiments with mice it would appear that the use of a single highly specific strain as a vaccine, such as was employed in the field experiment of 1936–7, is unlikely to confer satisfactory protection against other specific strains. The non-specific " master " strain might be expected to have more chance of success because it would evoke an immunological response of wider range. Here, however, we are in a dilemma, for the non-specific strain Bur, in some ways ideal for vaccinating, is only active to one-hundredth or less of the titre of the specific strain WS. If it should prove true that even specific strains contain a little of the other antigens, a higher titre specific vaccine such as WS might actually contain more of several components than a low titre " master " strain such as Bur. Possibly a high titre " intermediate " strain like the PR8, used by Francis and Magill (1937) and by Stokes *et al.* (1937) may prove more satisfactory than either.

The advantage of broadening the response by mixing many strains together to make a vaccine may be largely off-set by the reduction by dilution of some particularly important component which such mixing would necessarily entail. It must also not be forgotten that the total bulk of virus protein in a vaccine is probably of very great importance. Whilst it is not certain that the original infectivity titre of a product is an index of the bulk of virus protein contained in it, all the evidence we have got so far points to that conclusion. Therefore the incorporation of strains of low titre in a mixed vaccine might be disadvantageous no matter how desirable the inclusion of their antigenic factors.

The ferret experiments, in contrast to those with mice, tend rather to minimize the importance of strain differences. Heterologous immunity consequent upon recovery from infection is usually at a level adequate for complete protection against large doses of virus directly instilled into the nostrils. It is true that it tends to wane rather more quickly than homologous immunity, but the experiments on the reinforcement of basic immunity suggest that this may be accomplished with heterologous vaccines almost as well as with homologous virus, and that reinforcement may be effective against virus strains not previously encountered by the animal. We have emphasized in previous publications our view that the convalescent ferret with residual basic immunity offers a closer parallel to the majority of human adults than do normal ferrets or mice; nevertheless it would be dangerous to attach too much importance to the few experiments with such partially immune animals. The immunity following infection fades at different rates in different ferrets so that with small numbers, strictly comparable groups are hard to obtain. What is needed is the comparative testing of many different vaccines in human volunteers prior to an extensive epidemic caused by several different strains of virus.

SUMMARY.

1. An antigenic analysis of 28 strains of influenza virus isolated from human cases has been made by means of cross-neutralization tests in mice.

2. Many antigenic components enter into the constitution of the various strains. Of these 4 appear to possess major significance; they were found in very different proportions in the different strains.

3. It was found possible to classify the strains, on the basis of their content of the 4 major antigens, into 3 main categories, namely, highly specific strains, relatively non-specific strains, and intermediate strains.

4. A strain of virus can be shown to absorb from a heterologous serum only those antibodies which correspond to the antigenic components of the absorbing strain.

5. Active immunization experiments with mice show that the protection conferred by vaccination with a single strain is most effective against the homologous virus. The efficacy of a vaccine against a heterologous virus is largely dependent upon the closeness of antigenic relation between the strains concerned.

6. Experiments with ferrets suggest that in animals with some basic immunity the response to vaccination may be less specific than in animals with no previous influenzal experience.

7. The practical difficulties of applying the results of animal experiments to human vaccination are discussed.

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RATE OF ABSORPTION OF CARCINOGENS AND LOCAL TISSUE REACTION AS FACTORS INFLUENCING CARCINOGENESIS.

P. R. PEACOCK AND STEPHAN BECK.

From the Research Department of the Glasgow Royal Cancer Hospital.

Received for publication July 13th, 1938.

DESPITE the large and increasing number of chemical carcinogens discovered within recent years, we still know very little of the essential process of carcinogenesis. The development of sarcoma in the connective tissue of susceptible animals certainly depends on multiple factors, in addition to the presence in the tissue of an adequate quantity of a carcinogen, and some of these factors can now be discussed.

The influence of the solvent used as a vehicle for the fat-soluble polycyclic hydrocarbons was brought home to one of us (P. R. P.) by some experiments on fowls injected with 1 : 2 : 5 : 6-dibenzanthracene dissolved in lard, as compared with subsequent results in which chicken fat was used as the solvent. Whereas, with lard as solvent, quantities of from 1 to 4 mg. of 1 : 2 : 5 : 6-dibenzanthracene induced tumours in about 50 per cent. of the fowls, when chicken fat was used no tumours resulted. In subsequent experiments 1 : 2 : 5 : 6-dibenzanthracene and 3 : 4-benzpyrene, dissolved in chicken fat, have been injected into groups of fowls on several occasions without the induction of a single tumour. At first sight it appeared that the solvent, lard, might itself have some carcinogenic property, as, in the original group of 31 fowls injected with 1 : 2 : 5 : 6-dibenzanthracene dissolved in lard into the right breast, lard alone was injected into the left breast, and in 4 birds out of 16 tumour-bearing birds, tumours occurred at both sites of injection.

Recently, Burrows, Hieger and Kennaway (1936) described tumours in rats injected with lard only, and this observation would seem to make it desirable to avoid the use of lard as a solvent when the carcinogenicity of chemicals is under investigation. A possible explanation of the disparity between the results in fowls where different solvents were used would seem to be that the carcinogen was able to act on proliferating tissue, developed as a reaction to the lard. Such proliferation is not excited by the injection of chicken fat into chickens.

In order to obtain results on a larger scale, and more rapidly, a number of experiments were undertaken by one of us (S. B.), in which 3 : 4-benzyprene was injected into the right flank of mice, as described below :

0·5 mg. powdered 3 : 4-benzpyrene was subcutaneously inoculated into 10 mice, through an incision in the right flank, under anæsthesia (Fig. 1).

This experiment was repeated with 0·75 mg. 3 : 4-benzpyrene, with similar results (2 tumours out of 15 mice surviving for four months).

0·5 mg. 3 : 4-benzpyrene in 0·1 c.c. ether was subcutaneously injected into each of 20 mice (Fig. 2).

1 mg. 3 : 4-benzpyrene, in 0·2 c.c. mouse-fat, was subcutaneously injected into each of 8 mice (Fig. 3).

1 mg. 3 : 4-benzpyrene, in 0·25 c.c. olive oil, was subcutaneously injected into each of 17 mice (Fig. 4).

1 mg. 3 : 4-benzpyrene, dissolved in 0·25 c.c. of a mixture of $\frac{2}{3}$ olive oil and $\frac{1}{3}$ paraffin (melting-point 56° C.) was subcutaneously injected into each of 13 mice (Fig. 5).

1 mg. 3 : 4-benzpyrene, in 0·1 c.c. mouse lipoids, containing fats and sterols, was subcutaneously injected into each of 20 mice (Fig. 6).

The experiments fall into two groups : firstly, experiments in which 3 : 4-benzpyrene was injected in such a way that absorption and elimination was complete within about three to four months ; and secondly, experiments in which the absorption of the injected 3 : 4-benzpyrene was delayed beyond five or six months. In the first group tumours were rare, and in the second group they were very common. Details of the experiments are given in graphic form in the charts, Figs. 1 to 6. The charts show the lifetime of each mouse as a dotted line, and the time from the date of clinical diagnosis of a tumour to the death of the animal as a triangular expansion. Animals were examined at intervals of a week to a fortnight, and were killed as soon as it was obvious that a progressive tumour was growing. The charts, therefore, show the length of time that a tumour was clinically obvious before the animal was killed, but give no indication of the rate of growth. No purpose would have been served by allowing the animals to die of their tumours, as the object of the experiment was to ascertain, rather, the presence or absence of benzpyrene in the tissues at the time of the inception of new growth. The " + " sign at the end of the line indicates that fluorescence in the tissues was positive. " N " indicates that fluorescence was absent.

The immediate fate of the injected 3 : 4-benzpyrene was followed by examining the animals in the ultra-violet beam, and observing the fluorescence of the injected material after various time intervals. In this way it was possible to establish the following facts :

Solid benzpyrene, applied to connective tissue by open operation, rapidly goes into solution in the fatty tissues, and elimination is carried on steadily throughout the period of about three months necessary for complete elimination of the carcinogen.

Benzpyrene, dissolved in ether (violet fluorescence), is rapidly precipitated after its subcutaneous injection as finely divided solid benzpyrene (yellow fluorescence), and soon after goes into solution in the fatty tissues, as in the case of the solid benzpyrene described above (violet fluorescence).

It will be seen, therefore, that the first three methods of introducing benzpyrene, namely, powdered benzpyrene, ether solution and mouse fat solution all amount to solution of benzpyrene in mouse fat, the first two in the fat of the animal itself, and the third in heterologous mouse fat.

In the second group of experiments the solvents used tend to be retained at the site of injection, and also render the absorption of benzpyrene slower, as absorption can only occur from the surface of contact between the injected

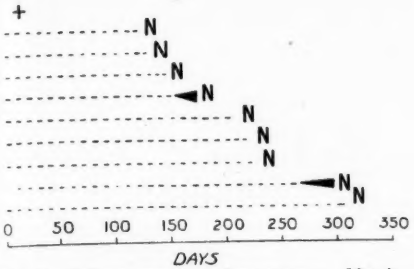

FIG. 1.—0·5 mg. powdered benzpyrene. 10 mice.

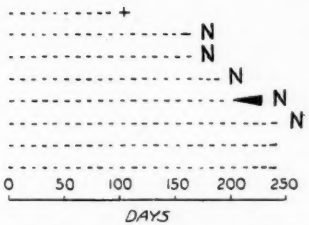

FIG. 3.—1 mg. benzpyrene in 0·2 c.c. mouse fat.

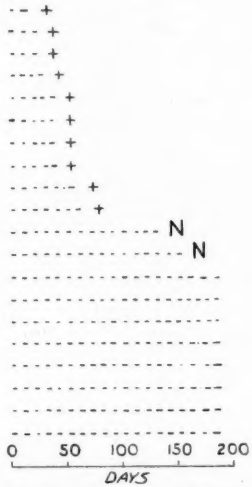

FIG. 2.—0·5 mg. benzpyrene in 0·1 c.c. ether. 20 mice.

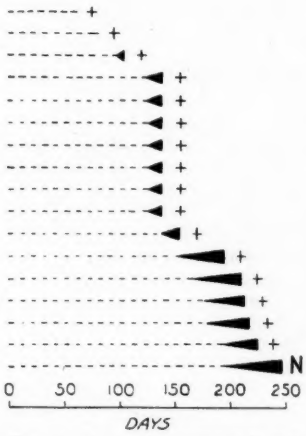

FIG. 4.—1 mg. benzpyrene in 0·2 5 c.c. olive oil. 17 mice.

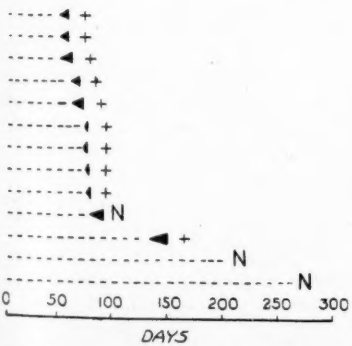

FIG. 5.—1 mg. benzpyrene in a mixture $\frac{2}{3}$ olive oil, $\frac{1}{3}$ paraffin. 13 mice.

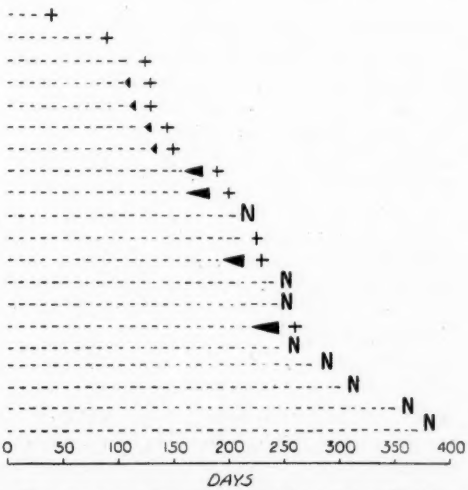

FIG. 6.—1 mg. benzpyrene in 0·1 c.c. mouse lipoids. 20 mice.

solvent and the living tissues of the mouse. Absorption is presumably effected by a continous process of partition of the benzpyrene between the injected solvent and the mouse's tissues and body fluids. It will be noted that, in the first group, where fluorescence tests showed benzpyrene to be completely eliminated in about three to four months, tumours were uncommon (5 out of 41 surviving for four months), while, in the second group, where benzpyrene was detectable for six months or more after injection, tumours were very common, (34 out of 46 surviving for four months). It appears, therefore, that retention of the carcinogen at the site of injection is of paramount importance, and outweighs any effect of tissue reaction to the vehicle used as solvent.

It is evident from the fact that fluorescence, due to unchanged benzpyrene, is still present after tumour growth has occurred, that only a part of the inoculated benzpyrene is used in these experiments in the induction of sarcoma,

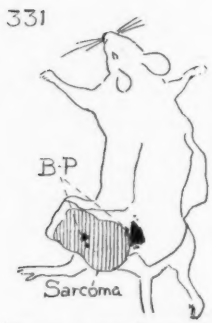

FIG. 7.—1 mg. benzpyrene in 0·1 c.c. mouse lipoids. 180 days after the injection.

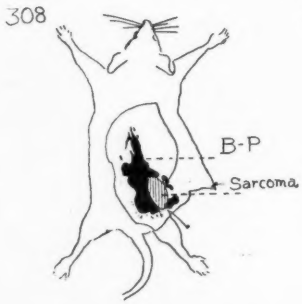

FIG. 8.—1 mg. benzpyrene in 0·25 c.c. of a mixture of olive oil $\frac{2}{3}$, paraffin $\frac{1}{3}$. 126 days after the injection.

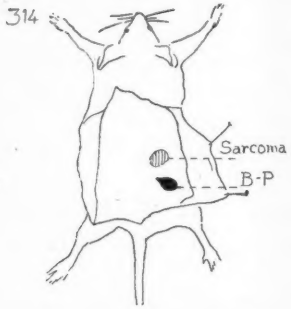

FIG. 9.—1 mg. benzpyrene in 0·25 c.c. of a mixture of olive oil $\frac{2}{3}$, paraffin $\frac{1}{3}$. 164 days after the injection.

and there is reason to think, from our own and other workers' results, that the quantities of benzpyrene employed (from $\frac{1}{2}$ to 1 mg.) are greatly in excess of the minimum quantity that will induce carcinogenesis, under favourable circumstances.

It is to be noted that in the majority of early tumours treated, as shown by typical illustrations (Figs. 7 to 9), the location of the fluorescence does not correspond with that of the tumour. There is usually a space of a few millimetres of apparently non-fluorescent tissue between the two sites. A similar observation was made in the study of fowl tumours, where tar was used as the carcinogen (Peacock, 1933). This observation suggests that in the course of absorption of the carcinogen a point of optimum concentration is automatically established, and that if this optimum concentration can be maintained for a sufficient length of time, then new growth results. It would further seem that there is a latent period, during which susceptible cells, subjected to the optimum concentration of a carcinogen, may recover, as in the first group of experiments, and this latent period for the connective tissue of the mouse appears to be about three months.

SUMMARY.

1. The induction of sarcoma in the connective tissues of mice, following the injection of 3 : 4-benzpyrene in various solvents, depends more upon the rate of absorption of the benzpyrene than upon the early local tissue reaction to the solvent.

2. When benzpyrene is eliminated within three months, tumours are rare (5 out of 41 surviving for four months); when elimination is delayed beyond six months, tumours are common (34 out of 46 surviving for four months).

3. Tumours occur at a point of optimum concentration of benzpyrene, which in these experiments is found to be a few millimetres away from the principal locus of benzpyrene.

One of us (S. B.) is working under a full-time grant from the International Cancer Research Foundation.

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ON THE FAILURE OF ACUTE AND SUBACUTE INFLAMMATION TO INFLUENCE CARCINOGENESIS WITH 3 : 4-BENZPYRENE.

STEPHAN BECK.*

From the Research Department of the Glasgow Royal Cancer Hospital.

Received for publication July 13th, 1938.

The object of these experiments was to examine the influence of acute and subsequent subacute inflammation on the reaction of the connective tissues of mice to 3 : 4-benzpyrene. An attempt was made to answer the following five propositions :

(1) Whether or not acute inflammation influences the rate of absorption of 3 : 4-benzpyrene.

(2) Whether or not the proliferation of the subacute inflammatory reaction tissue accelerates the development of sarcomas.

(3) Whether or not the sarcoma starts from the subacute inflammatory fibrous tissue.

(4) Whether or not the 3 : 4-benzpyrene acts at the locus of the injection or on the tissues in the neighbourhood through which it passes in the course of absorption.

(5) Whether the site of sarcomatous new-growth is determined by an optimum concentration of the carcinogen at some point or points along the route of absorption, or whether it is determined only by the greater susceptibility of certain cells to the carcinogen.

* Working under a full-time grant from the International Cancer Research Foundation.

Lacassagne (1929) and Vincent and Lacassagne (1933) produced inflammatory lesions in rabbits by the induction of abscesses and the injection of diatomaceous earth, and irradiated these lesions afterwards with x-rays. They observed malignant tumours on the site of the inflammatory lesions. Similar observations were made by Burrows, Mayneord and Roberts (1937), who injected kaolin and finely powdered silica, suspended in olive oil, into rabbits, and afterwards irradiated the site with x-rays. The conclusion is drawn by these authors that inflammation concentrates and retains the carcinogenic agent.

On the other hand, Berenblum (1935) was able to inhibit tar tumours and dibenzanthracene tumours in the skin of mice with dilute solutions of the irritants, mustard gas and its relatives, and with cantharidin.

A similar effect was found by Reimann and Hall (1936) with the application of para-thiocresol, which reduced the incidence of skin tumours in mice when applied to the skin together with dibenzanthracene. These authors point out that the sulphydryl group, which is contained in para-thiocresol, stimulates proliferation, differentiation, and organization, whereas dibenzanthracene de-differentiates and disorganizes. Cramer (1929) showed that cauterizing the base of a tar papilloma accelerated the onset of malignancy. Orr (1934) produced subcutaneous scar tissue by introducing linen thread sutures under the skin. The resulting fibrosis hastened carcinogenesis produced by painting with dibenzanthracene.

METHODS.

Preliminary experiments were carried out to find the dosage and concentration of turpentine, dissolved in olive oil, which produced a non-perforating abscess, and it was found that a 30 per cent. solution produced abscesses which did not perforate and which were absorbed in about four weeks. The solution of 30 per cent. turpentine in olive oil was therefore chosen, and the histological appearance twelve days after its injection was as follows : The corium contained a cyst which was traversed by interlacing fibrous trabeculæ. The tissues inside the walls of the cyst were completely necrotic. The walls were lined with extremely pyknotic and degenerate cells, which might have been polymorphonuclear leucocytes, but their morphology could not be distinguished. Outside this layer there was a zone of fibrosis.

Such abscesses were produced in groups 1, 2 and 3, while groups 4 to 6 served as controls.

Group 1.—Each of 15 mice received 1 mg. benzpyrene, dissolved in 0·25 c.c. of a sterile solution of 30 per cent. turpentine in olive oil (Fig. 1).

Group 2.—Each of 15 mice received 0·25 c.c. of a sterile solution of 30 per cent. turpentine in olive oil. All these mice developed abscesses. After three days the contents of the abscess were removed through a needle inserted obliquely into the cavity through the neighbouring tissues, to avoid ulceration and infection. The removed contents were replaced by 1 mg. benzpyrene, dissolved in 0·1 c.c. mouse lipoids. These mouse lipoids were extracted from dried mouse tissues, and contained sterols and fats (Fig. 2).

Group 3.—In each of 20 mice a sterile abscess was produced by 0·25 c.c. solution of 30 per cent. turpentine in olive oil. The injection was placed as exactly as possible halfway between the axilla and the groin. Three days later

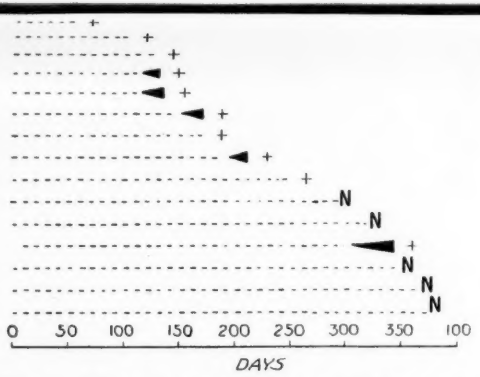

FIG. 1.—1 mg. benzpyrene in 0·25 c.c. solution of 30 per cent. turpentine in olive oil. 15 mice.

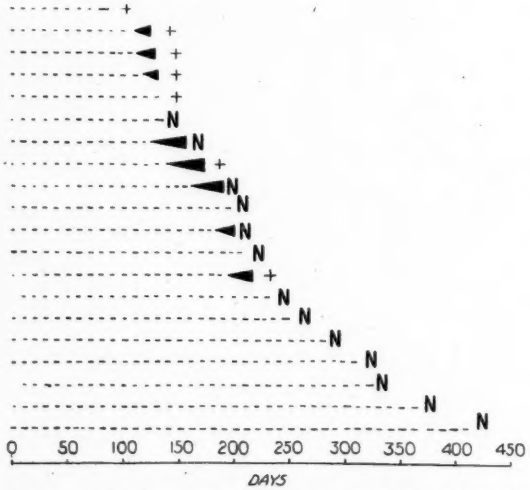

FIG. 3.—1 mg. benzpyrene in 0·1 c.c. mouse lipoids injected near the turpentine abscess. 20 mice.

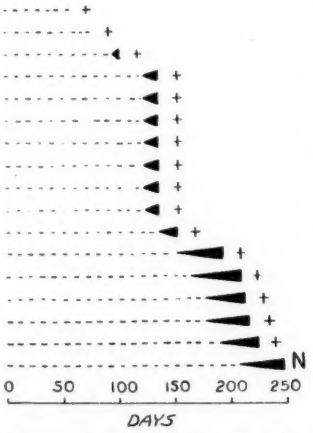

FIG. 4.—1 mg. benzpyrene in 0·25 c.c. olive oil. 17 mice.

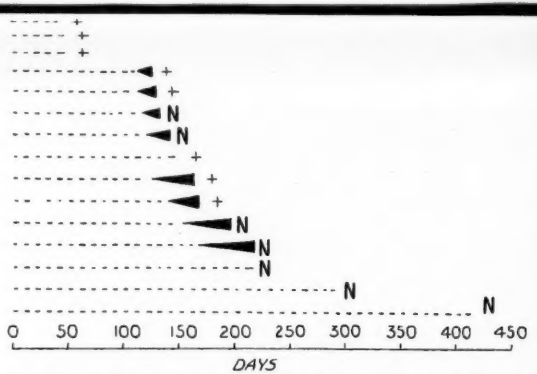

FIG. 2.—1 mg. benzpyrene in 0·1 c.c. mouse lipoids injected into the abscess. 15 mice.

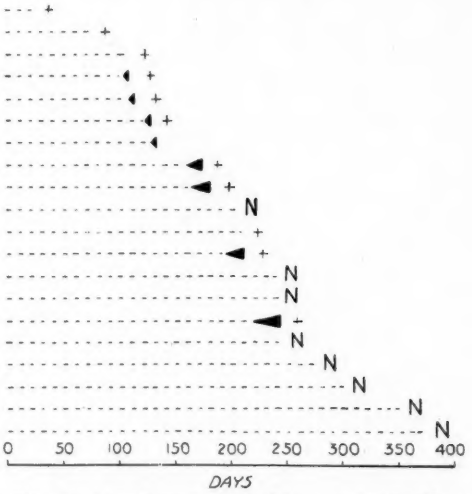

FIG. 5.—1 mg. benzpyrene in 0·1 c.c. mouse lipoids. 20 mice.

1 mg. benzpyrene, dissolved in 0·1 c.c. mouse lipoids, was injected 2–3 mm. nearer to the groin (Fig. 3).

Group 4.—Each of 17 mice was injected with 1 mg. benzpyrene, dissolved in 0·25 c.c. olive oil, as controls (Fig. 4).

Group 5.—Each of 20 mice was injected with 1 mg. benzpyrene, dissolved in 0·1 c.c. mouse lipoids, as controls (Fig. 5).

Group 6.—Each of 7 mice was injected with 0·25 c.c. solution of 30 per cent. turpentine in olive oil, as controls.

All these mice were examined every fortnight, and were killed soon after the tumour became definitely palpable. The tumours were histologically examined by Dr. L. Woodhouse Price, and all found to be sarcomas—two fibrosarcomas and the remainder the usual spindle-celled sarcomas. The site of injection was observed in the ultra-violet beam of a quartz mercury lamp, the light of which was filtered through Wood's glass. Under the ultra-violet beam the persistent benzpyrene appears as a spot of violet fluorescence, and the rate of absorption could be gauged by this method.

RESULTS.

The charts show the lifetime of each mouse as a dotted line, and the time from the date of clinical diagnosis of a tumour to the date on which the animal was killed as a triangular expansion. The " + " sign at the end of the line indicates that fluorescence in the tissues was positive; " N " indicates that fluorescence was absent. Group 6 showed neither tumours nor fluorescence. Every group which was injected with benzpyrene developed sarcomas, the groups which had abscesses as well as the controls, and most of them showed a fluorescent spot of persistent benzpyrene for six months. Early tumours were never found at the fluorescent spot itself, but always at a distance of 1–3 mm. from this spot.

The fluorescent tissue of one mouse was dissolved in caustic soda, extracted with alcohol, and the fluorescence spectrum was taken. The spectrum showed bands from (4080–4180) A and from (4300–4400) A. The slight shift of the first band towards the longer wave-length is probably due to additional products of the tissue in the solvent.

In group 3, in which benzpyrene was injected near the abscess, the tumour was never found at the locus of the turpentine injection, halfway between axilla and groin, but, with one exception, near the fluorescent spot on the opposite site from the locus of the turpentine injection.

DISCUSSION.

The foregoing experiments show that acute inflammation has no obvious effect on the action of benzpyrene. Neither is elimination increased, nor carcinogenicity suppressed. The subsequent fibrous reaction does not accelerate the appearance of sarcomas, and the fibrous tissue is not the starting-point for sarcomas. All sarcomas except one were found near the fluorescent spot of the persistent benzpyrene, but in the opposite direction from the locus of the turpentine injection. The tumours were always found at a small distance from the persistent benzpyrene. The whole area inside the cysts of the turpentine

abscesses, into which the benzpyrine was injected in groups 1 and 2, was completely necrotic. There were no living cells left in intimate contact with the benzpyrene from which a sarcoma could arise. The tissue of the neighbourhood only, through which the benzpyrene was slowly absorbed, could respond to the carcinogenic agent, and this is, in fact, what occurred.

The fact that the benzpyrene which is being absorbed induces the sarcoma, and the fact that the sarcoma arises always at a distance from the locus of the injection, allow, it is concluded, that sarcoma arises at points through which an optimal concentration of benzpyrene passes for an optimal time. In the tissue there are probably only a few points which fulfil this condition. Benzpyrene is thus titrated automatically against the tissues, in the course of its absorption, and this process determines which of the susceptible cells shall become malignant.

SUMMARY.

Mice were injected with 3 : 4-benzpyrene, dissolved in a 30 per cent. solution of turpentine in olive oil. A sterile abscess was thus induced around the benzpyrene. In other groups benzpyrene was injected into or near such an abscess. Two groups, injected with benzpyrene in the same solvents but without turpentine, served as controls. In another control group abscesses were induced but no benzpyrene injected.

All groups injected with benzpyrene developed tumours, and showed the fluorescence of persistent benzpyrene in the tissues for about six months.

The following conclusions are drawn :

1. Acute inflammation has no apparent influence on the rate of absorption and on the carcinogenicity of 3 : 4-benzpyrene in fatty solvents.
2. The inflammatory fibrous tissue does not accelerate or retard the development of sarcoma.
3. Sarcomas did not originate from the fibrous tissue of subacute inflammation.
4. 3 : 4-benzpyrene does not act at the locus of the injection, but on the tissues in the immediate neighbourhood through which it passes on its way to absorption.
5. 3 : 4-benzpyrene titrates itself against the tissues, and an optimal concentration for an optimal time determines which of the susceptible cells become malignant. Probably only a few points in the tissue fulfil these conditions.

I am indebted to Dr. P. R. Peacock for his interest, and to Dr. L. Woodhouse Price for pathological reports.

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THE SUSCEPTIBILITY OF VOLES TO HUMAN AND BOVINE STRAINS OF TUBERCLE BACILLI.

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From the Sir William Dunn School of Pathology, University of Oxford.

Received for publication July 26th, 1938.

The vole (*Microtus agrestis*) is infected in nature with a disease closely resembling tuberculosis (Wells, 1937). The infecting organism is a *Mycobacterium* whose taxonomic position is not yet settled. It has already been shown (Griffith, 1937) that " voles are highly susceptible to bovine tubercle bacilli, and that they can also be infected with human tubercle bacilli, though apparently with less certainty than with bovine bacilli ".

The experiments described in this paper were done in order to find out if the difference of susceptibility of the vole to human and bovine tubercle bacilli is sufficiently marked for the animal to be used to differentiate the two types.

TECHNICAL DETAILS.

Four strains of human and four strains of bovine tubercle bacilli have been used. The human strains are as follows :

1. T.B. Human Sherman, isolated in 1935 by, and received from, Prof. L. P. Garrod.
2. T.B. Human Lyons, isolated in 1918 by Dr. Stanley Griffith, and received from the National Collection of Type Cultures.
3. T.B. Human Scot. 489, isolated in 1937 by, and received from Dr. Stanley Griffith.
4. T.B. Human Smithburn 3104, received from Prof. R. A. Webb.

The bovine strains are as follows :

1. T.B. Bovine origin unknown, received in 1935 from the National Collection of Type Cultures.
2. T.B. Bovine No. 1 Bovine Virus, isolated in 1914, received from the National Collection of Type Cultures.
3. T.B. Bovine 34 B.V., isolated by, and received from Dr. Stanley Griffith.
4. T.B. Bovine Smithburn 36, received from Prof. R. A. Webb.

In all cases the appropriate dose of bacilli was injected into the peritoneal cavity. The dose was graded according to moist weight of bacilli. The growth from a 28–32 day culture on Dorset's egg medium was removed and ground up with 0·5 c.c. of distilled water in an agate mortar for 30 minutes. From this, serial dilutions were made.

The voles used throughout the experiment, were from the stock in the Bureau of Animal Population, which have been bred in captivity for some years.

* Working with a grant from the Medical Research Council.

EXPERIMENTAL.

The experiments described are divided into three series.

Series 1.

The first series of experiments were done to find out if voles can be infected with human and bovine tubercle bacilli, and, if so infected, whether there is any difference in the nature of the infection. Twelve voles were injected with doses varying from 1 mg. to 0·01 mg., 6 with human and 6 with bovine bacilli. None of these animals was killed. The last survivor lived for 81 days. The 2 voles which were injected with 1 mg. of human bacilli died after 59 and 81 days. At autopsy one showed enlarged caseous lymph-glands in the mesentery and at the bifurcation of the aorta; the other showed enlarged caseous lymph-glands in the mesentery and mediastinum with one small tubercle at the apex of the left lung. The other 4 voles, two of which received 0·1 mg. and the other two 0·01 mg. of human bacilli, died after 19, 30, 55 and 57 days. None of these showed any tuberculous lesions at autopsy.

The 6 voles which were injected with bovine bacilli died at intervals from 4–60 days. Two which died after 4 and 17 days showed no macroscopic tuberculous lesions. The other 4 which survived for 26–60 days all showed extensive tuberculosis at autopsy. The lymph-glands throughout the peritoneal cavity were greatly enlarged and wholly caseous. There were tubercles in the spleen, intestines and lungs. The mediastinal lymph-glands were much enlarged and caseous. In one instance there were caseous areas in the suprarenal glands.

This preliminary series suggested that the vole is very much more sensitive to bovine than to human bacilli. The results with human bacilli were quite inconclusive, but suggested that progressive disease may follow the injection of a large inoculum. No difference except one of degree was noted between the lesions following a large injection of human bacilli and those following an injection of bovine bacilli.

Series 2.

The results of the second series are set out in tabular form, but some explanation of the table is necessary. It will be noticed that in the first two experiments with human and bovine bacilli the number of voles dead in less than one month after injection is very high. It was not considered likely that the injection was the cause of these deaths. At this time a large number of stock voles (uninjected) died in the isolation laboratory, the percentage of deaths being no higher in the injected than in the uninjected. The cause of death was not immediately apparent, but was clearly not due to the tubercle bacilli injected. This trouble with what might be called normal deaths has, we hope, been largely overcome by attention to details of diet and housing. A description of these details is not pertinent to this paper. It is, however, as well to point out that the vole is a short-lived animal, and any experiments done with them should be done with two or more animals so that incidental deaths may be discounted.

Series 2.

Strain.		Number of voles injected.	Number of voles dead less than 1 month after injection.	Inoculum. 1 mg.	0·1 mg.	0·01 mg.	0·001 mg.	0·0001 mg.	0·00001 mg.
Human	Sherman	12	5	—	1/0	—	3/0	3/0	—
	N.C.T.C. Lyons	24	18	2±	1/0	1/0	2/0	—	—
	Scot. 489	24	4	4±	3/0	4/0	2/0	3/0	4/0
	Smithburn 3104	11	1	2± 1/0	4/0	3/0	—	—	—
Bovine	Vollum N.C.T.C.	6	4	—	—	1/+++	1/+++	—	—
	N.C.T.C. No. 1 Virus	24	16	2/+++	—	3/+++	—	3/+++	—
	34 B.V.	24	6	—	3/+++	4/+++	3/+++	4/+++	4/+++
	Smithburn 36	12	3	—	—	—	4/+++	2/+++	3/+++

All voles which survived were killed one month after injection and a post-mortem examination was made. The period of one month was chosen because it was found in the first series that at this time progressive tuberculosis can easily be demonstrated in those voles which were injected with bovine bacilli.

The figures in the table refer to the number of voles remaining after one month which had received the dose stated at the top of the column. The signs " 0 ", " ± " and " +++ " refer to the degree of infection found at autopsy. Thus " 0 " means that no infection was found ; " ± " means that a pin-point aggregate of bacilli was found embedded in a small white area of caseous material in the mesentery ; while "+++ " means that extensive and spreading infection was present. It was found that microscopically bacilli may be demonstrated even when no macroscopic lesions are present.

Those voles which died less than one month after injection have been discarded from the figures appearing in the columns of the table. Although some of the voles which received bovine tubercle bacilli showed disease less than one month after injection, no disease was found in those which received human tubercle bacilli. It was thought advisable to standardize the time elapsed after injection and to describe the lesions found at that period.

The post-mortem appearance of a vole in which progressive disease is present one month after injection is as follows : There is usually a small caseous lesion in the subcutaneous tissue where the needle passed at the time of injection. The lymph-glands in the peritoneal cavity are greatly enlarged and caseous. There is a line of caseous glands running the whole length of the abdominal aorta from the liver to the bifurcation of the aorta. The lymph-glands in the mesentery are much enlarged and caseous, as also are the glands near the pelvis of the kidney, at the hilum of the liver and the base of the spleen. There are usually tubercles on the peritoneal surface of the intestines and in the substance of the spleen. There may be macroscopical tubercles in the liver. The suprarenal glands are not usually affected. The mediastinal lymph-glands show a characteristic appearance. They are situated at the apex of the thorax, and are enlarged, hard and caseous. There may be small, grey, discrete tubercles in the lungs. The lymph-glands of the neck usually contain caseous patches. In all the lesions tubercle bacilli are very numerous, the caseous glands sometimes having the appearance microscopically of a homogeneous mass of acid-fast bacilli.

Results of Series 2.

The dose of human tubercle bacilli which will cause progressive disease in voles appears to be about 1 mg. Any dose less than 1 mg. has failed to produce any signs of infection in the 43 voles which survived one month and were then killed.

Those voles which received bovine bacilli showed disease in striking contrast to those which received human bacilli. The 37 voles which survived one month and were then killed all showed extensive disease at post-mortem, even when the infecting dose was as small as 0·00001 mg. It appears, then, that the vole is at least 100,000 times as sensitive to bovine tubercle bacilli as it is to human tubercle bacilli.

Series 3.

The results recorded in series 2 suggested that 0·001 mg. of culture of tubercle bacilli injected intraperitoneally into voles should determine the type of tubercle bacillus injected. Accordingly 10 voles received 0·001 mg. of human tubercle bacilli, strain Smithburn 3104, and another 10 received 0.001 mg. of bovine tubercle bacilli, strain Smithburn 36. These voles were all killed one month after injection. All those which received bovine bacilli showed extensive tuberculous disease similar to the condition already described in series 2. None of those which received human bacilli showed any macroscopic signs of disease.

SUMMARY.

1. The amount of human tubercle bacilli which will produce demonstrable disease after intraperitoneal injection in a vole after one month is at least 1 mg. Extensive and progressive disease has resulted one month after injection in all the voles which were injected with bovine tubercle bacilli in amounts varying from 1 mg. to 0·00001 mg.

2. It is suggested that a dose of 0·001 mg. moist weight of culture of tubercle bacilli injected intraperitoneally into voles should distinguish unfailingly between human and bovine tubercle bacilli in one month.

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THE SPECIFIC CHARACTER OF THE STAGE OF AGGREGATION IN AGGLUTINATION AND PRECIPITATION OF ANTIBODY-ANTIGEN COMPOUNDS.

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BORDET'S (1899) conception of the mechanism of immune serum agglutination of bacteria postulates a two-phase phenomenon in which the first phase corresponds to the *specific* combination of agglutinin with bacteria, and the second phase to the aggregation of the agglutinin-sensitized organisms in a manner analogous to the flocculation of certain colloidal suspensions in the presence of an electrolyte. In its present-day acceptation this theory implies, briefly, that bacteria or antigenic particles when mixed with the homologous immune serum become coated with specifically adsorbed "antibody globulin" which, passing out of solution, forms a layer of hydrophobic globulin on the surface of the organism. When this layer is complete, the particle, so far as its surface properties are concerned, reacts merely as a particle of physically

altered globulin, not exhibiting any specific character related to the antigenic structure of the underlying organism. All such globulin-coated organisms, irrespective of their antigenic structure, will cohere to form aggregates if brought into contact with one another; but such intimate contact may be prevented by the existence on all of the particles of a surface electrical charge of the same sign, usually negative, causing mutual repulsion. In the presence of an electrolyte, the electrically active ion of opposite sign reduces the charge on the particles below a critical level and permits effective contacts. According to this view, the stage of aggregation is wholly physical and non-specific, so that antigenically unrelated particles sensitized with their own antibodies will cohere when brought into contact with one another and form heterogeneous aggregates.

The more recent hypothesis advanced by Marrack (1934), which is supported by the work of Heidelberger and Kendall on immune serum precipitation of polysaccharide, azoprotein and crystalline protein antigens (1935*a*, *b*, *c*), supposes a continuous physico-chemical reaction in which the aggregates are built up of molecules of antigen and antibody specifically linked together to form a *lattice* structure in a manner analogous to the formation of crystals. From this hypothesis, therefore, it would follow that only antigenically related particles and their corresponding antibodies can enter into the formation of common aggregates; and when suspensions of antigenically unrelated particles, sensitized with their homologous antisera, are mixed together, the different kinds of particles will not combine together to form heterogeneous aggregates, as supposed in the Bordet theory, but each kind will independently form its own homogeneous aggregates.

Experimental evidence in support of the lattice hypothesis was also put forward by Topley, Wilson and Duncan (1935), who found that when mixtures of two different species of bacteria, morphologically distinguishable from one another and antigenically unrelated, were sensitized with their homologous antisera in such proportions as to ensure synchronous agglutination of the two types, the resulting clumps, observed microscopically, were homogeneous of one or other type. In disagreement with this observation, Abrahamson (1935) found that mixtures of sheep erythrocytes and Friedländer bacilli, agglutinating in the presence of their homologous antisera, formed both homogeneous and heterogeneous clumps; and Hooker and Boyd (1937), using a mixture of mammalian and avian erythrocytes with their respective antisera, found that when the serum proportion was optimal or in excess of optimal for the cell quantity, and agglutination of the two kinds of cells was synchronous, clumps containing both kinds of cells were formed. Hooker and Boyd regard the formation of heterogeneous clumps as strong evidence in support of the theory of non-specific aggregation, but the occurrence of homogeneous clumps as less conclusive. It is difficult to find an explanation of these apparently conflicting observations, but, in view of the experimental evidence to be put forward in the present paper, it may be significant that the only tests in which bacterial agglutination alone was used were those of Topley, Wilson and Duncan.

The composition of the aggregates formed may be inferred by comparison of the times taken for a particular stage of agglutination, observed macroscopically, in a mixture of two antigenically different agglutinating systems,

reacting synchronously, and in independent controls of each of these systems diluted with isotonic saline to give a concentration equal to that of the same system in the mixture. As the velocity of agglutination depends upon the incidence of effective contacts between the agglutinable particles, and therefore increases directly with the concentration of these particles, it follows that if all of the particles in the mixture react as a single agglutinating system and form heterogeneous clumps, the velocity of flocculation will be proportionate to the *total* concentration of particles, and will be much greater in the mixture than in the independent system controls in which the concentration of agglutinable particles, following dilution, will be only half the total of the mixture; but if the two systems in the mixture react independently, the comparable concentrations of the agglutinating systems in the mixture and in the controls will be equal, and agglutination in the mixture will not show acceleration. Carefully arranged experiments on these lines have been made by Hooker and Boyd (1937), who used the *precipitation* technique with the antigenically unrelated hæmocyanins of species of *Limulus* and *Fulgur* and their homologous antisera. The antigens, set up in a series of falling concentrations, were titrated against constant amounts of the homologous antisera. Each precipitating system was put up separately, in series, for comparison, tube by tube, with a mixture of the two, and the time of appearance of "visible flocculation" was recorded. The results showed, in most cases, accelerated particulation in the mixture as compared with the corresponding tubes in the independent system control series; in one example the times were seven minutes for the mixture and ten and a half minutes for each of the corresponding controls. The authors' contention that these results point to non-specific aggregation is well founded, but it cannot be said that any of the experimental evidence put forward excludes the possibility of a coincident *specific* action in aggregation. In the present paper an attempt will be made to advance evidence supporting the concept of *primary* specific aggregation.

EXPERIMENTAL PROCEDURE AND OBSERVATIONS.

The experimental work to be described covers immune serum agglutination of flagellated and non-flagellated bacteria, precipitation of yeast extract and crystalline protein antigens, and also the non-specific agglutination of bacteria by dialyzed, salt-free serum.

Tests Involving the H Form of Agglutination Only.

The types used were *Bact. typhosum*, *Bact. paratyphosum* A, *Bact. abortus equi*, *Bact. typhi murium* and *Bact.* Dar es Salaam. In the experiment illustrated in Table 1 the first three types mentioned were used. These were unrelated in their H antigens and were completely agglutinable by their own H antibody sera. Uniformly dense suspensions of the three organisms were prepared, and a preliminary titration was made of the homologous immune sera to determine the proportion of each, slightly in excess of the antibody optimum (Duncan, 1937), necessary to ensure synchronous agglutination in the three systems. The actual test was arranged to provide three synchronously

reacting controls, I, II and III, each containing one of the suspensions and its homologous immune serum in the same serum/suspension ratio and the same individual concentration as the corresponding system in the test mixture, IV; and three other controls, V, VI and VII, corresponding to I, II and III respectively and having the same serum/suspension ratio but *three* times the concentration of the agglutinating system. The test mixture, IV, contains the same concentration of each individual agglutinating system as the corresponding control I, II or III, but a *total* concentration of the mixture of all three equal to the concentration of the individual controls V, VI and VII. If, therefore, non-specific aggregation with formation of heterogeneous clumps composed of all three types occurs in the test mixture, the velocity of agglutination should be equal, approximately, to that in the controls V, VI and VII, and be much greater than that in I, II and III; but if, on the other hand, aggregation is strictly specific to each type, the velocity of agglutination will correspond with that in I, II and III, and will be much less than that in V, VI and VII.

Test-tubes of 9·0 mm. diameter were used and the reagents were measured in 0·5 c.c. units, each tube containing a total volume of 3·0 c.c. Observations were taken while incubating at 48° C. in a glass-sided water-bath. Owing to the uncertainty of determining accurately the moment of occurrence of some arbitrary stage of agglutination in suspensions of different densities, the method was adopted of recording the progress of agglutination at fixed intervals of time. To avoid the use of a multiplicity of symbols representing different stages in the progress of agglutination, italic numbers from *1* to *8* are employed. Thus the earliest trace of aggregation, just perceptible with the aid of a hand lens, is represent by *1*; a more advanced trace by *2*; a + trace by *3*; a + v.s. by *4*; a + s. by *5*; the larger flocculi + and ++ by *6* and *7*, and +++ with sedimentation by *8*.

The results of this experiment show that the agglutination times of the test mixture, IV, agree closely with those of the three controls, I, II and III, and are, roughly, three times as long as those of the controls V, VI and VII. This, as discussed above, indicates clearly that agglutination of the three reacting systems in the mixture, IV, was independent and specific, and there is no evidence of any interaction between these different systems.

In controls I, II and III, *pooled* sera were used to obtain a fairer comparison with the test mixture, IV, and it may be thought that the addition of the two heterologous sera would augment the agglutination by the homologous serum; this matter was kept in view, and it can be said that, at the serum dilutions used, no detectable effect on velocity of agglutination resulted. Strictly comparable results were obtained when isotonic saline was substituted for the two heterologous sera.

As the test mixture, IV, contains three independently reacting systems, it should be expected that the particles of any two would interfere to some extent with the rate of contacts between the particles of the third system, causing some retardation of agglutination. This effect is scarcely shown in Table 2, but it was appreciable, although never great, in other tests with flagellated or non-flagellated bacteria, when three or four different systems were mixed.

TABLE 1.—*Test with the H Form of Agglutination.*

	Diluted sera 0·5 c.c. unit vols.	Suspensions 0·5 c.c. unit vols.	Saline diluent 0·5 c.c. unit vols.	Time in minutes. 5. 10. 15. 20. 25. 30. 35. 40. 45. 55. Stages of agglutination (in italics).
I	1 *T*, 1 *E*, 1 *A*	1 *t*	2	— *1* *2* *3* *5* *5* *6* *6* *7*
II	1 *T*, 1 *E*, 1 *A*	1 *e*	2	— — *2* *2* *4* *5* *6* *7* *7* *8*
III	1 *T*, 1 *E*, 1 *A*	1 *a*	2	— — *2* *2* *3* *5* *6* *7* *7* *8*
IV	1 *T*, 1 *E*, 1 *A*	1 *t*, 1 *e*, 1 *a*	..	— — *1* *2* *3* *5* *6* *7* *7* *8*
V	3 *T*	3 *t*	..	*1* *5* *6* *7* *8* *8* *8* *8* *8* *8*
VI	3 *E*	3 *e*	..	*2* *6* *7* *8* *8* *8* *8* *8* *8* *8*
VII	3 *A*	3 *a*	..	*1* *6* *7* *8* *8* *8* *8* *8* *8* *8*

The three suspensions, *Bact. typhosum*, *Bact. abortus equi* and *Bact. paratyphosum* A are indicated by the letters *t*, *e* and *a* respectively, and the corresponding immune sera by the capitals *T*, *E* and *A*.

Every tube contains six 0·5 c.c. unit volumes.

The concentrations of the three suspensions, *t*, *e* and *a*, were 30 millions of organisms to 1·0 c.c. in controls I, II and III, and 90 millions in controls V, VI and VII.

Tests Involving the O Form of Agglutination Only.

With the O form of agglutination much less difficulty was experienced than with the H form in getting well-synchronized agglutination in suspensions of the different types and in recording accurately the stages of the reaction. The antigenic types employed were *Bact. typhosum*, *Bact. paratyphosum* A, *Bact. typhi murium* and *Bact.* Newport, in which the H antigens had been inactivated, and *Bact. dysenteriæ* Flexner Z and *Bact. pullorum*. The tests were made in a similar manner to those involving the H form of agglutination, but smaller volumes of the reagents were used in 5 mm. agglutination tubes. Altogether twenty tests were made, using mixtures of 2, 3 or 4 different organisms with a variety of arrangements of the controls, and in all the results were harmonious.

The inferences to be drawn from the results shown in Table 2 are similar to those offered in the case of Table 1. Regarding the mixture as two independently reacting types, the velocity of agglutination, calculated from the time taken, is exactly proportionate, with one minor exception, to the concentration of the suspension concerned. The very sharp readings shown are attributable to the slowness of the reaction and the wide spacing of the time intervals in this test, but they are consonant with the results of all similar tests. Invariably the antigenically unrelated agglutinating systems in a mixture appeared to react as independent systems, and, in one case in which,

TABLE 2.—*Test with the O Form of Agglutination.*

	Diluted sera 0·25 c.c. unit vols.	Suspensions 0·25 c.c. unit vols.	Saline diluent 0·25 c.c. unit vols.	Time in minutes. 20. 30. 40. 50. 60. 70. 80. 100. Stages of agglutination (in italics).
I	1 *A*	1 *a*	2	— *1* *3* *4* *5* *6* *7* *8*
II	1 *N*	1 *n*	2	— *1* *3* *4* *5* *6* *7* *8*
III	1 *A* + 1 *N*	1 *a* + 1 *n*	..	— *1* *3* *4* *5* *6* *7* *8*
IV	2 *A*	2 *a*	..	*4* *5* *7* *8* *8* *8* *8* *8*
V	2 *N*	2 *n*	..	*3* *5* *7* *8* *8* *8* *8* *8*

Experiment with suspensions (H antigens inactivated) of *Bact. paratyphosum* A and *Bact.* Newport; represented by *a* and *n* respectively and their homologous immune sera *A* and *N*.

The concentrations of the suspensions were, in I and II, 250 millions of organisms in 1·0 c.c., in III, 250 millions of each in 1·0 c.c., and in IV and V, 500 millions in 1·0 c.c. Incubation temperature 50° C.

after a synchronous start, agglutination progressed more rapidly with one system than with the other, it was possible to observe the appearance, simultaneously, of two different stages of aggregation in the mixture.

Tests with Precipitation Reactions.

Tests were made at first with two antigenically different yeast extracts and their homologous precipitating sera, following the technique already described for the agglutination experiments. The results of these tests suggested the operation of some non-specific factor, apparently in addition to the specific element, in precipitation. A larger series of tests was then made with crystalline hen egg albumin and crystalline human serum albumin antigens and the homologous precipitating sera. The antigen solutions were arranged to contain uniform concentrations of the crystalline proteins, and the dilutions of the homologous immune sera were adjusted to give synchronous precipitation of these two antigens at antibody/antigen ratios well in excess of the constant-antibody optimum of Dean and Webb (1926).

The results of the precipitation experiment given in Table 3 differ from those of the agglutination tests in showing an acceleration of particulation in the test mixture, III, as compared with its constituent systems separately, in controls I and II, which suggests an appreciable degree of non-specific aggregation in the mixture. In fact, if consideration be confined to I, II and III, the results, when adjusted for comparison, agree closely with those recorded by Hooker and Boyd (1937) from their experiments with the hæmocyanin antigens. On the other hand, when comparison is made between III and the controls IV and V, each containing, presumably, the same concentration of *nuclei of aggregation* as the total concentration in III, the slower particulation in III does not support the concept of a stage of aggregation *wholly* non-specific.

Comparing I and II with IV and V, it will be seen that the velocity of particulation (read as the inverse of the time taken) is in the ratio I or II : IV or V : : 1 : 2, and is proportional to the concentration of the precipitating system. The velocity of particulation in the test mixture III is not related

TABLE 3.—*Test with the Precipitation Reaction.*

	Diluted sera 1·0 c.c. unit vols.	Antigen dilutions 1·0 c.c. unit vols.	Saline diluent 1·0 c.c. unit vols.	Time in minutes. 3. 6. 8. 11. 14. 17. 21. Stages of precipitation (in italics).
I	1 *M*, 1 *E*	1 *m*	1	0_1 0_3 *1* *3* *5* *6* *8*
II	1 *M*, 1 *E*	1 *e*	1	0_1 0_3 *1* *3* *5* *6* *8*
III	1 *M*, 1 *E*	1 *m*, 1 *e*	..	0_2 0_4 *3* *5* *7* *8* *8*
IV	2 *M*	2 *m*	..	0_4 *3* *6* *8* *8* *8* *8*
V	2 *E*	2 *e*	..	0_4 *3* *6* *8* *8* *8* *8*

Crystalline human serum albumin, *m*, and crystalline hen egg albumin, *e*, and the homologous precipitating sera, *M* and *E* respectively.

9 mm. tubes were used and the total volume in each tube was four 1·0 c.c. unit volumes. Incubation at 48° C.

The stages of increasing opalescence preceding visible particulation are represented by 0_1 to 0_4, and particulation by the italics *1* to *8* as before.

directly to the concentration of its constituent systems singly or in combination, but shows an increase over the velocity of I or II in the ratio of, roughly, 3 : 4.

In a repetition of this experiment it was found that on increasing the concentration of the controls I and II in the ratio 3 : 4 (the concentrations in III remaining as before), the stages of particulation in these two tubes became synchronous with those in III. So that the increase in velocity of particulation in the mixture III, attributable to a non-specific factor, was comparable to the effect of increasing the concentration of one of its constituent controls in the ratio 3 : 4. The ratio 3 : 4, in this connection, has occurred frequently in similar tests and it may have some significance, but it must be admitted, in the case of Table 3, that the time intervals of reading are too short to exclude the possibility of error arising from differences in the times of reading the five tubes and, for this reason, the figures should not be accepted as *precise*.

In order to exclude any possible effect of differences in the time occupied in the stage of " sensitization ", it was the practice, after agglutination or precipitation reactions were complete, to re-disperse the aggregated particles (to opalescence in the case of precipitates), and to record the progress of re-aggregation. The results of these second readings did not differ materially from those of the original readings, and only the latter have been recorded in this paper.

In another series of experiments with the crystalline albumin antigens, synchronously developing precipitates of each were prepared in bulk, collected without centrifuging and re-dispersed to opalescence in isotonic saline. An experiment was then set up corresponding to that illustrated in Table 3 with the test mixture, III, and the controls I, II, IV and V, the opalescent suspension of immune compound being substituted for the separate serum and antigen, and an equal volume of saline diluent added to controls I and II. By

adequate dilution of the whole experiment the time of particulation could be delayed so that it was feasible to take readings at much longer time intervals. The results of these tests were in harmony with those illustrated in Table 3, except that the acceleration attributable to the non-specific factor in the test mixture III was usually delayed, and the *earliest* readings suggested independent, specific aggregation, as in the agglutination tests ; but in every case the acceleration due to non-specific aggregation was well shown in the later readings. If, as will be suggested later, the non-specific factor in aggregation depends on the great preponderance of " antibody globulin " in the immune compound formed with soluble antigens, it is conceivable that a reduction in the proportion of antibody, due to the dissociation of some of it from the antibody-antigen compound, when dispersed in high dilution, would result in a lessening of the non-specific effect.

Tests of Non-Specific Agglutination by Dialyzed Serum.

In a serum dialyzed free from salt, proteins left in solution after precipitation of the " euglobulin " are in a state of physical instability, and a second precipitate usually forms when the serum is heated to 48°–55° C. After this a further precipitation will occur when certain salt-free colloidal suspensions, including dialyzed suspensions of *flagellated* bacteria, are added to the serum. It seems that protein adsorbed on the bacterial surface is immediately precipitated (possibly a mutual flocculation of two colloids), and the bacteria, thus coated with precipitated protein, are rapidly agglutinated. No attempt has been made in this work to determine the electrical conditions of the two colloids, but this does not seem necessary to explain the reaction. This kind of agglutination requires a relatively high concentration of the serum, as in the precipitation reaction, and the considerable amount of adsorbed protein, passing out of solution, causes a marked increase in the opacity of the suspension preceding agglutination. So far as the antigenic structure of bacteria is concerned the agglutination is wholly non-specific, and it seems to accord fairly well with the physical aggregation envisaged in the Bordet theory for the second phase of immune agglutination, except in regard to the role of an electrolyte. Salt-free non-specific agglutination follows the rule of optimal proportions of bacteria to serum protein, and is quickly prevented by an excess of bacteria and delayed by an excess of serum. It is therefore not necessary to invoke the lattice hypothesis to explain inhibition in excess of either reagent.

Non-flagellated bacteria were not agglutinated even at the highest serum concentrations used.

After heat precipitation (50° C.), dialyzed serum when titrated with acid will yield a further precipitate about the iso-electric zone of the proteins, and the titre of non-specific agglutination of the serum rises sharply as the iso-electric zone is approached. Under such conditions non-flagellated bacteria are also agglutinated. Acid titration was not used in the present work.

The titre of non-specific agglutination may also be increased by adding to the dialyzed serum a small amount of salt—an equivalent volume of $M/400$–$M/100$ NaCl solution—but further additions of salt progressively lower it,

until agglutination is prevented when a sufficient quantity has been added to stabilize the concentration of protein present.

Although salt-free serum agglutination is non-specific, a considerable specific absorption of antibody will occur if a dialyzed *immune* serum is used in fairly high concentration with the homologous organism, and it seems possible that such absorbed "antibody globulin" may have an adjuvant effect on the non-specific protein agglutination. For this reason, only immune sera heterologous to the bacteria, or *normal* rabbit sera, were used in the experiments to be described.

Table 4 gives the result of an experiment in non-specific agglutination following the arrangement of the tests recorded in Tables 1 and 2. For a fair comparison two separate dialyzed sera were used, although similar results may be got with a single serum or a pool of sera. A preliminary test was made of a number of different dialyzed sera and suspensions to find two sera which at a fixed dilution would give synchronous agglutination of equally dense suspensions of two antigenically unrelated organisms, when each serum was tested with the two organisms separately and each organism with the two sera. To obtain this degree of synchronization presented some difficulty, but success was achieved with two dialyzed and heated normal rabbit sera, designated α and β, and suspensions of flagellated *Bact. typhosum* and *Bact. abortus equi*.

TABLE 4.—*Test with Non-specific Aggregation.*

	Dialyzed sera 1·0 c.c. unit vols.	Dialyzed suspensions 1·0 c.c. unit vols.	Distilled water 1·0 c.c. unit vols.	Time in minutes. 3. 6. 9. 12. 15. 18. 21. 25. 30. Stages in agglutination (in italics).
I	1 α	1 *t*	2	*1 2 3 4 5 5 6 7 8*
II	1 β	1 *e*	2	*1 2 2 3 4 5 6 7 8*
III	1 α + 1 β	1 *t* + 1 *e*	..	*3 5 6 7 8 8 8 8 8*
IV	2 α	2 *t*	..	*3 5 6 7 8 8 8 8 8*
V	2 β	2 *e*	..	*3 4 6 7 8 8 8 8 8*

Non-specific agglutination of dialyzed suspensions of *Bact. typhosum*, *t*, and *Bact. abortus equi*, *e*, by dialyzed *normal* rabbit sera, α and β.

This table is self-explanatory, and shows clearly the effect of elimination of the specific element in agglutination in the test mixture, III, in which the velocity of agglutination, equal to that in the controls IV and V, may be accepted as proportionate to the total concentration of the two antigenically unrelated organisms. It may be noticed that the velocity ratios of controls IV and V to controls I and II show some variation, and are not so closely correlated to the corresponding densities of the suspensions as in the immune serum agglutination tests. This could, to some extent, be corrected by increasing the serum ratios in I and II; however, this comparison is less important than that between the test mixture and IV and V. The results of this purely non-specific aggregation should be compared with those of specific agglutination in Tables 1 and 2, and of the mixed effects in Table 3.

SUMMARY AND DISCUSSION.

It is not the intention of this paper to consider the actual structure of aggregates of immune compounds, but, by observation of the relation of velocity of aggregation to the individual and total concentrations of the different aggregable particles in a mixture of two or more antigenically heterogeneous reacting systems, to adduce evidence bearing on the question of whether aggregation may be attributed, primarily or wholly, to specific combinations of homogeneous antigenic types, in accordance with the lattice hypothesis put forward by Marrack, or to purely physical and non-specific combination, possibly involving heterogeneous types, as envisaged in the Bordet theory and supported by the recent discussions of Hooker and Boyd.

The work on immune serum agglutination of bacteria, illustrated in Tables 1 and 2, shows that in a mixture of two or three antigenically heterogeneous agglutinating systems, reacting synchronously, the velocity of agglutination is that of the individual systems and not that of a combination of the systems. These and similar experiments furnished unequivocal support to the view that agglutination by immune serum is *specific* in all its stages.

On the other hand, the results of all precipitation tests with yeast polysaccharide and crystalline albumin antigens pointed to a non-specific aggregation, possibly superadded to a primary specific one. When comparison is made between the velocity of particulation in a mixture of equal volumes of two antigenically different, synchronously reacting, precipitating systems, and separate controls of these two systems diluted to a concentration equal to that of each in the mixture (that is, equal to half the *total* concentration of the mixture), the relative acceleration of particulation in the mixture points clearly to some degree of common aggregation of particles of the two systems, but it is not evidence of a *wholly* non-specific aggregation. Again, when comparison is made between the mixture and the undiluted separate system controls having a concentration equal to the total concentration of the mixture, the slower particulation in the mixture shows that aggregation of the primary antibody-antigen particles is not wholly non-specific, and, to this extent at least, a specific factor would seem to be admissible. The relation of the ratio *velocity of aggregation/total concentration* in the test mixture to the similar ratios in the controls confirms the view that in precipitation reactions aggregation involves both specific and non-specific combinations. However, it may be contended, from analogy to the immune serum agglutination of bacteria, that the *primary* mechanism is specific, and this contention is supported by the results of tests with mixtures of antigenically unrelated immune precipitates dispersed in isotonic saline and allowed to re-aggregate, when the acceleration of particulation attributable to non-specific combination followed a period in which the reaction was apparently specific.

Adopting the lattice hypothesis of immune compound aggregation, which is supported by the tests of immune serum agglutination of bacteria, the occurrence of a non-specific effect in the analogous precipitation reaction may, perhaps, be explained by the great preponderance of antibody globulin in the immune compound formed with a soluble antigen, as contrasted with the relatively small proportion of antibody necessary to agglutinate bacteria.

This high proportion of combined hydrophobic protein may render the primary specific aggregates susceptible to some degree of non-specific, physical aggregation comparable to the non-specific aggregation of the heavily protein-coated bacteria referred to in Table 4, or to the flocculation of other protein-coated colloid particles.

In conclusion, the view that aggregation of the immune compound is specific in character, as demanded by the lattice hypothesis, is supported by the results of the agglutination reactions with immune serum, and, although the effect of a non-specific factor in aggregation was never absent from the precipitation reactions, its incidence seems to be secondary to a specific combination, and the results of these tests may be accepted as not inconsistent with the lattice hypothesis.

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MICROSCOPICAL OBSERVATIONS ON BACTERIOPHAGE OF *BACT. COLI*.

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THE lytic action of bacteriophage has been studied microscopically by many workers, but the appearances described do not agree owing to the different methods used. The work has been reviewed on a number of occasions. Topley and Wilson (1936) state that this disagreement is due to the fact that the observations were not always made "under the best modern conditions of microscopy and microphotography".

Observations with transmitted light and examination of stained specimens (Wollmann, 1925; Bronfenbrenner, 1927; Bronfenbrenner and Muckenfuss,

1927) obtained at intervals from lysing cultures failed to give detailed information, since phage bodies are not visible in transmitted light or in stained preparations.

Dark-ground illumination has revealed more information, but since the ordinary slide and coverslip technique has been used the results have been unsatisfactory. Da Costa Cruz (1926) observed swelling of bacteria and polar bodies in some cases. Bronfenbrenner, Muckenfuss and Hetler (1927) saw swelling of bacteria but not bursting. D'Hérelle (1926) described granulation, swelling and bursting of the bacteria, and gives the number of released phage bodies as 15 to 25.

Bechhold and Villa (1925) claimed to show phage bodies with their gold method. Burnet (1933) gave a photomicrograph taken by J. E. Barnard with ultraviolet light of bodies in a filtrate of a lysed culture. D'Hérelle (1938) expressed doubt whether the phage bodies, owing to their small size, can be seen and photographed.

In view of the unsatisfactory methods previously used, it was considered that the improved microscopical technique for the demonstration of the internal structure of bacterial cells (Merling-Eisenberg, 1935*a*) would be of value in observing and photographing bacteria before, during and after lysis.

Technique.

An object slide 0·9 mm. thick with a hollow-ground cavity about 0·3 mm. deep was filled with liquid paraffin and a coverslip carrying a hanging drop broth culture was placed on it (Merling-Eisenberg, 1928*a*). In this way a hanging-drop culture is made available for ordinary dark-ground illumination. Satisfactory cultivation can then be carried out for prolonged periods on the warm stage of the microscope at 37° C. (Merling-Eisenberg 1928*b*). For particulars of the optical equipment previous publications are referred to (Merling-Eisenberg, 1935*a*, *b* ; 1937*a*, *b*, *c*.).

Experiments on Bact. coli.

Hanging-drop cultures of *Bact. coli* inoculated with bacteriophage $1 : 10^{-7}$ were prepared as described above and observed continuously.

Within three hours most of the bacilli became motionless and a great number of thrown-off flagella could be seen. Whilst no particles could be detected in the sterile filtered broth used, a great number of particles of various sizes appeared in the cultures of *Bact. coli* as soon as the lysis had started.

An apparently normal bacillus of about 3μ length and 0·8μ breadth that had been moving freely in the medium may after a period of indefinite movement become motionless. Within the next hour fine granules begin to appear inside the bacillus (fig. 1), in some cases being in active movement, in other cases at first motionless. Simultaneously the size of the bacillus increases in all diameters to about 4μ in length and 1·3μ in breadth, though individual bacteria show wide differences. Nothing like this was ever observed in normal cultures of *Bact. coli* alone. After an interval that may vary between 5 minutes to 1 hour after the appearance of granules inside the bacillus, the actual lysis takes place in one of several ways. The lysis always occurs in a definite explosive

manner. The disruption may occur without any further change in the size or shape of the bacillus, as if the whole of its cell membrane suddenly became permeable like a sieve. On other occasions an opening appears at one end or at the side of the bacillus, through which the granules are released. In other cases the bacillus swells up still further or becomes completely spherical a few seconds before bursting. The granules that are released by the explosion show very distinctly as a cloud of very actively moving particles with a blue diffraction colour, thus producing the same phenomenon of selective diffraction as had been seen with viruses (unpublished). As far as experience goes at present, the way in which the disruption of the bacillus will occur cannot be foreseen from the appearance of the bacillus before the lysis. If the Numerical Aperture (N.A.) of the optical system is as low as 1·15, the granules appear as a cloud of ill-defined particles, but with an aperture of about 1·25 the particles are very nearly resolved.

Preliminary observations on lysing cultures showed that a distinction between unspecific particles and the specific granules—the supposed phage bodies—could only be made by observing the process of lysis in single bacilli. Two distinctive features of the phage bodies were found in the course of systematic observations: their blue diffraction colour and their fairly uniform size.

The phage bodies seem to be expelled from the bacillus by the force of the explosion to some considerable distance that is estimated at about 5 to 6 times the length of the bacillus. From one bacillus a number of phage bodies is released varying between 150 and 350 according to the size of the bacillus.

The phage bodies are in active Brownian movement and soon leave the vicinity of their "birthplace". If lysis is in full swing throughout the culture the number of phage bodies free in the medium soon reaches astronomical figures (fig. 3).

Simultaneously with the release of the phage bodies the bacillus breaks up more or less. The bits and pieces into which the "membrane" shrinks hang together for a while (fig. 2) and then disperse slowly. They consist of particles of various sizes forming rings, discs, etc., but always quite white in colour, in contrast with the blue phage bodies. This colour distinction has been maintained under the conditions of cultivation for as long as three weeks.

As is well known, bacteria develop internal granules as a result of age, particularly if the culture is kept at a temperature below 37° C. Some of these particles of age are fairly big in size, measuring round about 0·3μ, and are frequently arranged like polar bodies (fig. 5). A fine grain disintegration may

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EXPLANATION OF PLATE I.

Fig. 1.—Three colon bacilli showing phage bodies inside (3-hour culture). × 2500. Enlarged × 2.

Fig. 2.—The same field 5 minutes later. The phage bodies have been released; the bacilli have collapsed.

Fig. 3.—Phage bodies free in the medium.

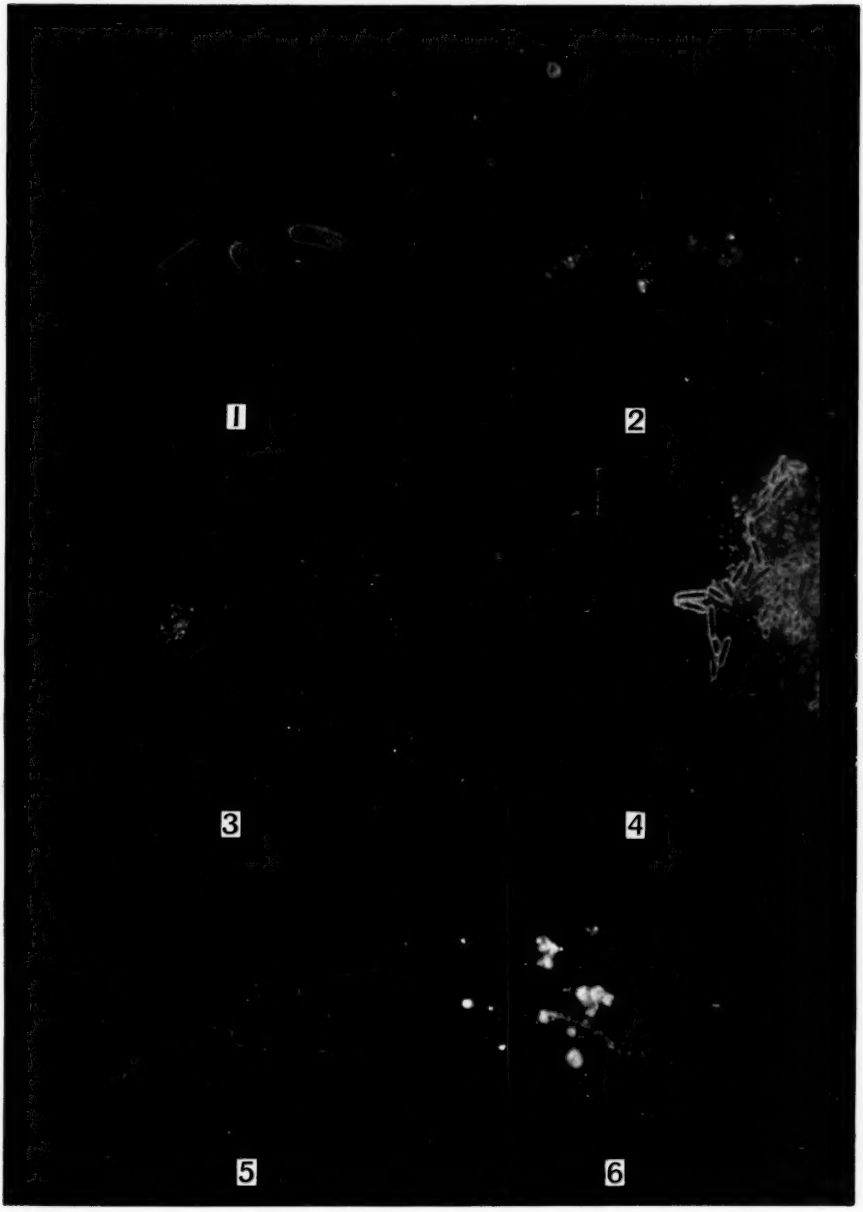
Fig. 4.—Resistant culture growing in spite of presence of abundant phage bodies.

Fig. 5.—Coarse grain disintegration of old culture.

Fig. 6.—Fine " " " "

In figs. 5 and 6 no phage bodies were present.

Figs. 3 to 6 are taken at an initial magnification of × 2500, not enlarged.



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be found as well in ageing cultures (fig. 6) that occurs without the presence of bacteriophage.

Since the phage bodies were found to be just beyond the limit of resolution, direct microscopical measurements were impossible. A photometer used in astronomy for measuring the size of objects beyond the limit of resolution of the telescope was adapted, and by this means the size of the phage bodies was found to be 25 mμ. This figure corresponds well with the size of 25mμ. given by Elford, Andrewes and Tang, (1936) and of 39mμ. given by McIntosh and Selbie (1937).

Prolonged observations were also made to observe the growth and fate of resistant colonies of *Bact. coli*. In several series of multiple observations on resistant colonies a number of bacilli which seemed resistant to the action of the phage in their youth were attacked later and lysis took place, but always some individuals of the resistant colony remained and appeared normal during the whole length of the observations (three weeks). Such resistant bacilli kept their size and shape all the time and never developed any phage bodies inside (fig. 4).

DISCUSSION.

The observations described above demonstrate the particulate nature of the bacteriophage of *Bact. coli*. Thus they point in the same direction as experiments of Elford, Andrewes and Tang (1936), and McIntosh and Selbie (1937), who have shown by centrifugalization that the bacteriophage action is bound to an agent of particulate nature. Due to their small size the phage bodies are not visible in transmitted light or stained preparations which were the methods used by Wollmann (1925). D'Hérelle (1926) has not observed any motion of the bodies within the bacterial cells, and he also reports only between 15 and 25 bodies released from one bacteria. This is no doubt due to the optical system which he has employed. It obviously enabled him to perceive only the coarser granulate disintegration of the membrane into 15 to 25 particles, but not the ten times greater number of the much fainter phage bodies.

SUMMARY.

The lytic action of Bacteriophage of *Bact. coli* has been observed and photographed in the following stages. Particles appear within the bacterial cell, which swells up and finally bursts, discharging the particles into the surrounding medium. The phage is distinguished from non-specific particles and its size found to be 25mμ.

My thanks are due to the Middlesex Hospital Medical School authorities and to the Sir Halley Stewart Trust for facilities enabling this work to be done.

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FUNCTIONAL HYPERPLASIA OF THE PARATHYROIDS IN EXPERIMENTAL ANÆMIAS.

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HYPERPLASIA of the parathyroids is commonly related to bone diseases, to renal insufficiency, or to grave disturbances of calcium metabolism such as are observed in sprue. The causal sequence is not always clear; thus, for example, in renal insufficiency, parathyroid hyperplasia may appear previous to the renal disorder or *vice versa*. The cause of parathyroid enlargement in renal diseases is supposed to be a disturbance of calcium metabolism caused by phosphate retention (Albright *et al.*, 1934, 1937). It seems that aside from the above-mentioned diseases cases of parathyroid hyperplasia are few. Gilmour and Martin (1937), who investigated some hundreds of cases, mention only a few cases in connection with disorders of internal secretion and one case of pernicious anæmia (see also Bergstrand, 1921; Castleman and Mallory, 1937). In this case of pernicious anæmia there was observed a marked hyperplasia without any concomitant bone- or renal disease.

The author, working on toxic hyperchromic anæmias, observed there extensive changes of the parathyroids. Rabbits were given hydroxylamin intravenously over a long period, and developed severe anæmias, alterations in hæmopoietic tissues, hyperplasia of parathyroids, but no changes in the bones (Eisler, 1937). The kidneys of such rabbits in most of the cases did not show any alterations which could be called morphological evidence for renal insufficiency or for any renal damage.

This paper will present data concerning the weight and structure of parathyroids of normal and anæmic rabbits.

Rabbits usually have four parathyroids, two of which are situated within the thyroids, the other two lying near by. The external parathyroids were removed shortly after death, carefully freed from connective tissue and weighed on an analytical balance to the nearest fifth of a milligram. Histological examination showed that some structural changes in the gland resulted from the separation. For this reason weighings were made on the isolated gland, while from the same animal another gland without removal of the peripheral tissue served for histological study. This does not modify the value of the results since, as Table I shows, there are no significant differences between the left and right external gland.

TABLE I.

No.	Body-weight (g.)	Parathyroids. Left side.	Parathyroids. Right side.
Normal Rabbits.			
1	2450	1·4	1·3
2	2680	2·5	2·1
3	3050	3·2	3·6
4	2200	2·8	2·4
5	2100	2·2	2·5
6	2370	2·5	2·6
Average mg.		2·4	2·4
Rabbits with Toxic Anæmia.			
1	2850	25·4	23·1
2	2960	20·6	19·6
3	2350	14·5	16·1
4	3010	9·6	10·2
5	3240	24·0	25·0
6	2670	12·4	13·0
7	2820	10·2	11·4
8	2910	19·6	18·2
9	3210	26·4	27·0
10	2290	15·3	14·8
11	2270	18·0	19·0
Average mg.		17·8	17·9

For comparison glands of 12 normal rabbits of the same body-weight, sex and race were taken. Table II presents the results, which show that normally the external parathyroid gland has a weight between 1·4–3·6 mg., with a mean value of 2·3 mg.

TABLE II.

Normal Rabbits.

No.	Body-weight (g.).	Parathyroid gland (mg.).	No.	Body-weight (g.).	Parathyroid gland (mg.).
1	2000	3·6	7	2450	1·4
2	2050	2·3	8	2680	2·5
3	1950	2·4	9	3050	3·2
4	2520	2·0	10	2200	2·8
5	2900	1·8	11	2100	2·2
6	2760	1·6	12	2370	2·5

Average mg. : 2·3.

TABLE III.

Rabbits with Toxic Anæmia.

No.	Body-weight (g.).	Parathyroid gland (mg.).	No.	Body-weight (g.).	Parathyroid gland (mg.).
1	2890	13·0	14	2960	17·8
2	3260	12·5	15	2300	22·1
3	3000	27·0	16	2850	25·4
4	2550	15·0	17	2960	20·6
5	2650	14·5	18	2350	14·5
6	2900	11·0	19	3010	9·6
7	3010	14·0	20	3240	24·0
8	2430	25·0	21	2670	12·4
9	2400	14·0	22	2820	10·2
10	2890	21·0	23	2910	19·6
11	3100	19·5	24	3210	26·4
12	3000	23·4	25	2290	15·3
13	2780	26·1	26	2270	18·0

Average mg. : 18·1.

Glands of anæmic rabbits whose anæmia was caused by hydroxylamin injection are increased in their weight. For these experiments there were only taken glands of animals whose kidneys did not show morbid changes. The animals either were bled to death or died of an intercurrent disease. The average weight of the parathyroid gland of anæmic rabbits is increased to 18·1 mg. The microscopical examination, which was carried out on formol or formol-mercuric chloride fixed material, showed a simple hyperplasia of the glandular cells. The glands were embedded in paraffin and cut in the longest axis. Results presented in Table IV indicate that anæmia by itself does not cause hyperplasia or increased weight of the parathyroids. The rabbits were made anæmic by repeated bleeding. The amount of red blood-corpuscles varied between 2,000,000–3,000,000, as in toxic anæmic rabbits.

TABLE IV.

Rabbits : Anæmia by Repeated Bleeding.

No.	Body-weight (g.).	Parathyroid gland (mg.).
1	2890	2·4
2	2760	2·2
3	3050	1·9
4	2360	3·1
5	2450	2·0

Average mg. : 2·3.

DISCUSSION.

As Gilmour and Martin showed, the weights of the parathyroids are elevated constantly only in those human cases in which diseases of the kidneys were present. Our results, supported by 26 observations, show that increase in the weight of parathyroids with simultaneous cellular hyperplasia might also occur in cases where no damages of the kidneys could be detected. The results of Gilmour and Martin are only in apparent contradiction to the present data, however. In kidney diseases of the type studied by Gilmour and Martin (nephritis and renal insufficiency), or by Bergstrand, it is usual to find more or less severe anæmias, whose characteristics place them among the toxic anæmias (Parsons and Ekola-Strolberg, 1933 ; Brown and Roth, 1922 ; Castle and Minot, 1936 ; Löwinger, 1938 ; Nordenson, 1938). Their origin is not yet clear, but it seems that toxic substances are retained and cause depression of hæmopoietic activity. Macrocytic anæmia with high colour index also occurs in sprue. A significant increase of the weight of parathyroids is also present. In sprue severe disturbances of the calcium metabolism appear, and the hyperplasia of the parathyroids is considered to be compensatory.

Some time ago it was widely held that the function of parathyroids is detoxication. This theory, advanced mainly by Noel Paton, has been replaced by the supposition that the only activity of the parathyroids is to govern the calcium metabolism (Shelling, 1935 ; Okkels, 1935).

The questions arise : What may be assumed to be the cause of the hyperplasia in cases of nephropathia, of experimental toxic anæmia, of sprue, etc ? Are disturbances of calcium metabolism the basic cause of the hyperplasia of parathyroids ? Or, do parathyroids have another function, still unknown, which is in relation to chronic poisoning or to hæmopoietic activity ? There seems to be no evidence supporting the assumption that hæmopoiesis and the parathyroids are in some way connected with each other. Complete removal of the parathyroids causes no anæmia. As said above, toxic substances cause anæmia either in nephropathia or in the present experiments on rabbits. These substances have in common, that they are to a certain extent convulsive agents. Becher and Litzner (1926) and others have shown that in cases of nephropathia with nitrogen retention, phenol or phenol-like substances appear

in the blood. These chemical compounds are typical convulsive agents. Hydroxylamin, used in our experiments, affects the central nervous system like derivatives of ammonia and produces typical cortical convulsions. Now, calcium is considered to be a substance which diminishes the effect of convulsive agents on the nervous system. There seems, thus, to be a possible connection between intoxication and calcium metabolism. The hyperplasia of parathyroids may then be secondary to certain changes of the calcium metabolism. These changes would in the present cases be connected with the detoxication of those convulsive agents, which are given experimentally (*e. g.* hydroxylamin), or produced by the diseased body in the clinical cases.

SUMMARY.

Data are presented, which show that a significant hyperplasia of parathyroids without concomitant renal damages occurs in rabbits in cases of toxic anæmia. The cause of this hyperplasia is discussed with respect to the relation between calcium metabolism and detoxication.

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THE ROLE OF THE ADRENAL GLAND AND OF THE RAISED METABOLISM IN THE PRODUCTION OF ORGAN HYPERTROPHY IN THE THYROID-FED RAT.

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IT has long been recognized that enlargement of the heart may occur in exophthalmic goitre ; the early descriptions of the disease by Parry, Basedow and Graves allude to this cardiac feature, a comprehensive description of which is to be found in the communication of Parkinson and Cookson (1931). That the enlargement is in some way dependent on the presence of an excess of the thyroid hormone in the circulation has been proved by the results of thyroid feeding in animals. Hoskins (1916) and Herring (1917) observed that not only the heart but other organs, such as the kidneys and adrenals, are enlarged by thyroid administration—observations which have since been confirmed by Hewitt (1920), Cameron and Carmichael (1920), Squier and Grabfield (1922), Simonds and Brandes (1930) and others.

The manner in which hypertrophy of the organs of the thyroid-fed animal is produced is still imperfectly understood. Hoskins (1916) believed the increased activity of the affected organs, resulting from the elevated metabolism, to be mainly responsible. Herring (1917), while not denying that increased metabolic activity might be a contributory factor, affecting principally organs such as the liver, believed that it does not satisfactorily explain the relatively greater increase in the weight of the heart and kidneys in the thyroid-fed as compared with the exercised animal. Having shown that the amount of adrenalin in the adrenals is increased by thyroid feeding, Herring, while admitting the absence of proof and not overlooking the possible importance of the endocrine system as a whole, was tempted to suggest the existence of a connection between the abnormal stores of adrenalin and cardiac hypertrophy.

In view of the uncertainty regarding the causation of the changes in the organs of the thyroid-fed animal, and because it has lately been suggested that the circulation-rate, and therefore the work of the heart, is proportional to the oxygen requirements of the tissues, it seemed to us desirable that the matter should be more closely investigated. The ability of the rat to survive bilateral adrenalectomy for considerable periods owing to the presence of accessory cortical tissue furnishes a means by which the role of the adrenal medulla in the process may be assessed, while the availability of drugs of the nitrophenol group, which increase metabolism in the manner of thyroid but, unlike the latter, leave the pulse-rate unaffected, enable the relative importance of increased oxygen consumption *per se* to be determined. We have applied

these methods to the study of the mechanism of organ hypertrophy in the thyroid-fed rat.

METHODS.

Growing male rats, bred in the Department from Wistar stock and weighing about 200 g., were used. They were maintained on a diet of a 3 : 1 mixture of dried milk and crushed wheat, each animal receiving a daily ration of 20 g. of the mixture made into a mash with water. This diet was from time to time supplemented by cabbage and cod-liver oil. It was adequate to produce healthy growth, and was well partaken of by all the animals with the exception of those receiving dinitrophenol; the latter were induced to consume their ration by adding 5 g. of anhydrous glucose to the daily feed. The following groups were compared: normal and adrenalectomized animals, with and without addition of thyroid to the diet, and animals fed with dinitrophenol.

Bilateral adrenalectomy was performed in one stage by the lumbar route under ether anæsthesia. A proportion of the animals succumbed in from 6 to 15 days after operation; the remainder made a good recovery. Thyroid feeding was commenced 5 to 10 days after the operation.

Desiccated thyroid B.P. was intimately incorporated with the food, beginning with 50 mg. per day for 2–3 days, raising it to 100 mg. for 6–15 days, and finally to 200 mg. In judging the dose we were guided by the animals' condition, their appetite and their weight, any tendency to rapid growth on the one hand, or to loss of appetite and sickness on the other, being countered by either increasing or decreasing the amount of thyroid administered. The experiments were continued for from 30 to 50 days. 2 : 4-dinitrophenol was given in a similar manner, commencing with 5 mg. per day for 2 days, then 25 mg. for 4 days, and finally 50 mg. for about 23 days. As in the case of thyroid it was sometimes necessary to vary the dosage according to the condition of the animals.

At the end of the experimental period the animals, together with the untreated controls, were killed with chloroform and their organs carefully dissected out and weighed.

RESULTS.

The weights of the bodies and organs of the rats are given in Table I. In all cases we have expressed the size of the organs in terms of the percentage of the body-weight on the day immediately prior to the commencement of thyroid or dinitrophenol feeding.

The conclusions drawn concerning the effect of the various procedures on the organ weights (with the exception of the liver and spleen) are based on the results of a statistical analysis, using the *t*-test.

Normal animals.—These animals, which were all healthy, thrived during the experimental period and gained an average of 20 per cent. in weight.

Adrenalectomized animals.—The growth of the adrenalectomized animals was not as rapid as that of the controls, mainly owing to the set-back following operation. The discrepancy in this respect in the two groups is not, however, sufficiently great to invalidate our conclusions. On comparison with the

TABLE I.—*Mean Weights of the Organs Expressed as the Percentage of the Initial Body Weight.*

The figures in brackets denote the group with which comparison was made in computing the *t* values.

	Body weight, gm.		Heart.		Kidneys.		Liver.	Spleen.	Thyroid.		Adrenals.		Testes.	
	Initial.	Final.	%.	*t*.	%.	*t*.	%.	%.	%.	*t*.	%.	*t*.	%.	*t*.
1. Normal (19 rats)	200	240	0·43	..	0·92	..	5·2	0·48	0·012	..	0·020	..	1·23	..
2. 2 : 4-dinitrophenol-fed (7 rats)	203	200	0·36	4·46* (1)	0·98	1·36 (1)	4·9	0·37	0·010	1·43 (1)	0·019	0·86 (1)	1·20	0·24 (1)
3. Thyroid-fed (18 rats)	210	205	0·52	4·70* (1)	1·12	4·97* (1)	5·7	0·50	0·009	2·82* (1)	0·026	3·27* (1)	1·07	2·00† (1)
4. Adrenalectomized (13 rats)	220	235	0·42	0·83 (1)	0·95	0·99 (1)	5·0	0·47	0·011	0·90 (1)	..	..	1·29	0·58 (1)
5. Thyroid-fed adrenalectomized (11 rats)	200	200	0·55	0·57 (3); 5·40* (4)	1·31	3·63* (3); 8·27* (4)	6·4	0·55	0·009	0·16 (3); 3·18* (4)	..	..	1·26	0·29 (4); 2·00† (3)

* $P = <0{\cdot}005$; unmarked values of *t* are not significant.
† $P = 0{\cdot}05$.

controls it is clear that adrenalectomy does not affect the weight of the heart, kidneys, thyroid and testes. That this should be so is somewhat surprising, but it is fortunate in that it enables us to assess with confidence the effect of thyroid feeding on the organs of adrenalectomized animals.

Thyroid-fed animals.—The effect of thyroid on the body-weight of an animal is to some extent dependent on the amount given ; in general small quantities increase, and large quantities decrease, the body-weight (Herring, 1917 ; Cameron and Carmichael, 1920). The doses we have used were large, and for this reason growth was brought to a standstill over the experimental period, despite hypertrophy of the organs.

In the main the changes produced by the thyroid hormone in the organs of our animals conform with those recorded by previous investigators. The heart, kidneys, adrenals, and to a less extent the liver and spleen, are hypertrophied ; the size of the thyroid is probably diminished, as was found by Hoskins (1916), Herring (1917), Hewitt (1918) and others. The mean weight of the testes is low, but it is doubtful whether there is really a significant decrease in the weight of these organs. It has been the uniform experience of other workers that the testes are moderately enlarged by thyroid.

Thyroid-fed adrenalectomized animals.—Comparison of the organ weights of the adrenalectomized animals which had received thyroid with those which had not, shows that those organs which are hypertrophied in the normal animal by thyroid are similarly affected even when the adrenal medulla has been removed. The kidneys are exceptional in showing a probably greater increase in the adrenalectomized animal. The unessential nature of the adrenal medulla in the causation of organ hypertrophy is again apparent when the effects of thyroid feeding on adrenalectomized and normal animals are compared.

From these results it is abundantly clear that the adrenal medulla is in no way concerned with the widespread effects of thyroid on the size of the organs of the rat.

Animals fed with 2 : 4-*dinitrophenol.*—In its effect on the body-weight in our series of animals this substance resembled thyroid. We were unable to measure the metabolism of these animals, but amounts of dinitrophenol considerably smaller than those used by us are known to produce a marked increase in the metabolic rate (Cutting, 1933), while we believe the absence of an increase in weight to be sound evidence of increased metabolism in animals which were eating well.

It is evident that none of the organs is hypertrophied by dinitrophenol. The heart, which in the thyroid-fed animal is very considerably enlarged, is actually significantly smaller ; the thyroid, adrenals and testes correspond to those of the normally-fed animal. It would seem, therefore, that merely by increasing the metabolism of an animal it is not possible to produce enlargement of the organs. We are thus enabled to draw the conclusion that the action of thyroid on the organs cannot be ascribed to its capacity for increasing the rate of metabolism.

It is of interest that the heart, which might have been expected to enlarge in response to the increased oxygen demands of the tissues, is actually smaller in the dinitrophenol-fed animals.

DISCUSSION.

In agreement with previous investigators we have found the heart, kidneys, liver, spleen and adrenals of the thyroid-fed rat to be enlarged. Our main object in studying this aspect of thyroid action was to determine whether the adrenals are concerned in these changes, especially in the case of the heart. Herring (1917) was tempted to correlate the large heart of the thyroid-fed animal with overactivity of the adrenals because in such animals these glands are enlarged and contain an increased amount of adrenalin. Further, he was of the opinion that the abnormal production of adrenalin is to be regarded as an attempt to antagonize the action of the thyroid hormone, since it appeared that those animals whose adrenals contained most adrenalin were best able to tolerate thyroid (Herring, 1915, 1917).

We obtained no definite evidence that the adrenals protect the animal from thyroid: when due allowance is made for the adverse influence of the operation, our adrenalectomized animals withstood the effects of thyroid as well as the intact animals. Also our experiments show quite clearly that the adrenals are not concerned in the production of organ hypertrophy. Thus there is no basis in fact for the suggestion of Herring (1917) that adrenalin is a factor to be reckoned with in this connection.

The mechanism of the hypertrophy of the heart in the thyroid-fed animal has never been clearly explained. Two reasons might be suggested for the increased cardiac output which occurs under the action of thyroxin: (i) a direct action of the hormone on the cardiac muscle, producing an increased rate of contraction and secondary hypertrophy; (ii) that the increased output is in some way related to the increased oxygen demands of the tissues. The latter view, that the cardiac output is principally conditioned by the metabolic needs of the tissues, which again are largely governed by the thyroid, has led to the advocacy of total thyroidectomy in conditions of congestive heart failure. It is suggested that by removal of the thyroid and reduction of the metabolic needs of the tissues, the failing cardiac muscle will be relieved of a proportionate amount of its ordinary work (Blumgart, 1933). In the light of our experiments with dinitrophenol we are satisfied that increasing the rate of general tissue metabolism does not, of itself, result in hypertrophy of the cardiac muscle. It has been shown by Cutting (1933) that dinitrophenol fed to rats in amounts considerably less than our own animals received leads to an increase in the basal metabolic rate of at least 50 per cent.; in our experiments the arrest in the weight increase of the dinitrophenol-fed animals was almost exactly equivalent to that of the thyroid-fed group, in spite of the fact that the former animals consumed the same rations and were receiving in the addition 5 g. of glucose. It would seem justifiable to conclude that the metabolism of the dinitrophenol-fed group must have been raised to at least the same extent as that of the thyroid-fed group. In spite of this the heart weight of the dinitrophenol-fed group is significantly smaller than that of the controls, and very markedly smaller than that of the thyroid-fed group. We conclude, therefore, that an increased rate of tissue metabolism *per se* does not lead to an increase in the bulk of the cardiac muscle and, by inference, does not increase the work of the heart.

Lewis (1931), Priestley (1931), Yater (1931) and their co-workers have shown that the excised and perfused heart of the thyroid-fed animal continues to beat at an enhanced rate for several hours. According to Markowitz and Yater (1932), cultures of the heart of the 2-day-old chick embryo, in which functioning nerve tissue and ganglia are undeveloped, pulsate more rapidly when thyroxin is added to the culture medium. It is clear, therefore, that the action of thyroxin on the rate of the heart-beat is independent of the general metabolism of the body, and that it is exerted directly upon the cells of the cardiac muscle. Though tachycardia and increase in the basal metabolic rate develop coincidentally, they arise from two distinct activities of the thyroid, and the increased rate of the heart is not the direct result of an increased demand for oxygen due to the raised metabolism of the body tissues.

It seems probable that the cardiac hypertrophy is secondary to the increased rate of contraction under the influence of thyroxin. The magnitude of the effort of the heart in thyrotoxicosis is readily apparent from the fact that whereas the minute volume of the resting normal adult is about 4 litres per minute, the minute volume of the heart under the influence of the thyroid hormone can, under the same conditions, be more than double this figure. This increase in the output of the heart is due, in the main, to the increase in the rate of pulsation, and we think it is to be expected that in time the cardiac musculature will hypertrophy under the strain, thereby reinforcing itself against the increased demands on its activity.

In regard to the effect of thyroid feeding in increasing the weight of the kidneys we have no explanation to offer. Though it might be suggested that the increased nitrogen excretion that results from thyroid feeding produces a work-hypertrophy, as in the case of high protein feeding, it is noticeable that dinitrophenol-feeding does not have this effect. It is conceivable that though both thyroid and dinitrophenol increase oxygen consumption, their mode of action on the metabolism is not, in fact, the same ; that whereas in the case of thyroid there is an increased protein catabolism, the same does not hold for dinitrophenol.

CONCLUSIONS.

1. The adrenal medulla is not concerned with the hypertrophy of the heart and other organs of rats fed with desiccated thyroid substance.

2. 2 : 4-dinitrophenol, which increases the rate of general tissue metabolism, does not produce hypertrophy of the organs of the rat.

3. Since increasing the rate of oxygen consumption of the tissues does not, of itself, result in cardiac hypertrophy, the effect of thyroid on the heart cannot be ascribed to this aspect of its function. Increased metabolism and tachycardia, although they develop simultaneously under the action of thyroid, are unrelated. It is probable that the hypertrophy of the heart is secondary to the increased rate of contraction and the extra work performed.

We wish to express our gratitude to Dr. W. R. S. Doll for the trouble which he has taken in the statistical analysis of our results. One of us (W. J. G.) is indebted to the Medical Research Council for a part-time grant during the period of this investigation.

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A STUDY OF EXPERIMENTAL IMMUNITY TO THE VIRUS OF PSITTACOSIS IN THE MOUSE, WITH SPECIAL REFERENCE TO PERSISTENCE OF INFECTION.

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OUR knowledge of immunity to the virus of psittacosis is as yet fragmentary. It is known, of course, that the animal recovering from experimental infection is immune, and some evidence has been produced (Bedson, 1933 ; Levinthal, 1935) that mice can be immunized with psittacosis virus inactivated by formalin. It has also been shown that as the result of infection or immunization with live virus specific antibodies are produced, though their titre in the circulation is never high and their demonstration by means of the neutralization test is, in consequence, unsatisfactory (Rivers and Schwentker, 1934). But we have no knowledge as to the durability of the immunity evoked by infection or the injection of inactive virus, nor are we familiar with its mechanism. Chance observations made by the author have suggested that persistence of infection after recovery is by no means uncommon with this virus and the occurrence of numerous carriers both amongst psittacines bred in captivity and in those in the wild state (Meyer and Eddie, 1933 ; Burnet, 1934 ; Burnet and Macnamara, 1936) supports this view. The phenomenon of persistent infection is known to occur with other viruses and one wonders how common it is in the case of psittacosis and what part it plays in ensuring the immune state. It was in the hope of elucidating some of these problems that the experiments, reported in this paper, were undertaken.

EXPERIMENTAL.

Active Immunization by Means of Formolized Virus.

Is this possible with inactive virus?

In a previous paper (Bedson, 1933) it was shown that formolized virus, incapable of producing infection in mice, would produce a considerable degree of active immunity in these animals and Levinthal (1935) reported that a high degree of immunity could be so obtained. Fortner (1936), however, has maintained that infection was essential to the production of immunity against psittacosis virus. In the author's earlier experiments (Bedson, 1933), as in those of Levinthal, no attempt was made to ascertain whether the formolized virus used for immunization was completely non-infective; particular attention has been paid to this point in the experiments now reported.

Preparation of vaccine.—A 5 per cent. suspension of virulent mouse spleen in M/50 phosphate buffer pH 7·6 was lightly centrifuged to remove the grosser tissue particles and formalin was added to a concentration of 0·1 per cent. The formolized suspension was left in the refrigerator (+ 4° C.) for 10–14 days before use. No attempt was made to find out the least time necessary for inactivation of the virus under these conditions.

Immunization of mice.—Mice were immunized by three intraperitoneal injections of 0·3 c.c. of the vaccine, spaced at weekly intervals.

Test of immunity.—This was carried out 10 days after the last dose of vaccine. It consisted of the intraperitoneal injection of varying doses of a highly active passage virus; 4 vaccinated mice and a similar number of controls were used for each dose.

Test for presence of live virus in the vaccinated mice.—This was done in the case of each batch of immunized mice in order to rule out the possibility that the formolized vaccine contained some living, but probably attenuated, virus and that its efficacy was due to its power to infect. Two mice for every 12 vaccinated animals were killed at the time of applying the test of immunity, and their spleens were examined for the presence of live virus. This examination consisted of the microscopic examination of spleen smears stained by Giemsa and Castañeda and the intraperitoneal injection of mice with a 5 per cent. suspension of the spleen. Two mice were so injected, the dose being 0·5 c.c. and 12–14 days later these mice were killed and examined in a similar manner. After the third passage in mice the investigation was discontinued; in no instance has live virus been recovered from vaccinated mice.

Results of immunity test.—The results of two experiments are resumed in Table I; they leave no doubt that a satisfactory immunity can be evoked in mice be means of a formolized vaccine containing no demonstrable active virus.

Is a vaccine made from the washed virus as good as that made from the crude virus suspension?

This point was next examined. The washed virus was prepared from a 5 per cent. suspension of virulent mouse spleen in phosphate buffer by differential centrifugation in an angle centrifuge; the virus was washed three times

TABLE I.—*Immunity Produced in Mice with Formolized Vaccine.*

3 doses of 0·3 c.c. Vaccine intraperitoneally at 7 days' interval; Injection of live virus intraperitoneally 10 days after last dose of vaccine.

Test-dose of virus. 0·5 c.c. intraperitoneally of 5 per cent. virulent mouse spleen suspension diluted:	Vaccinated mice.		Control mice.	
	1st experiment.	2nd experiment.	1st experiment.	2nd experiment.
10^{-2}	3/4	4/4	0/4	0/4
10^{-3}	4/4	3/4	0/4	0/4
10^{-4}	3/3	4/4	2/4	0/4

The numerator of the fraction denotes the number of mice surviving, the denominator the number tested.

and the final suspension consisted principally of elementary bodies. The virus content of the crude and washed virus suspension was ascertained by titration in the guinea-pig's skin; they were approximately the same. The two suspensions were treated with formalin in the manner described and batches of mice were immunized with the two vaccines, the procedure being the same as in the previous experiments. Test of immunity was made 10 days after the last dose of vaccine and the results are recorded in Table II.

TABLE II.—*Comparison of Immunizing Power of Vaccines Made from Crude and Washed Virus Suspensions.*

Test-dose of virus. 0·5 c.c. intraperitoneally of 5 per cent. virulent mouse spleen suspension diluted:	Mice immunized with—			
	Crude vaccine.	Washed virus vaccine.	*Nil.*	
10^{-2}	S. S. S. S.	S. S. S. S.	D. 7	D. 7 D. 8 D. 10
10^{-3}	S. S. S. S.	D. 11 S. S. S.	D. 9	D. 10 D. 16 D. 31
10^{-4}	S. S. S. S.	D. 16 D. 25 D. 26 S.	D. 10	D. 10 D. 11 D. 11

S. = survived. D. = Died, the number indicating the day after injection on which death occurred.

Although the findings are slightly in favour of the crude vaccine in this experiment, it is doubtful whether the difference is really significant. A second experiment gave a very similar result. It will be noted that those mice dying in the washed-virus vaccine group were not those which had received the largest test dose of virus. This has happened on other occasions during the course of this work, and it is somewhat difficult to explain except on the score of chance. In the two mice dying on the 11th and 16th days after the test inoculation, the post-mortem appearances were those of psittacosis and virus was present. In the two remaining mice which died on the 25th and 26th day the appearances were not those of chronic psittacosis and no virus could be found microscopically. There was, however, no other obvious cause of death in these mice, and it has, therefore, been attributed to psittacosis. It might be pointed out here that in experiments of this kind the finding of virus in the tissues of mice dying after the receipt of the test dose of live virus does not necessarily mean that they have died of psittacosis, since virus is to be found equally readily in the survivors which have not been obviously ill. This point will be dealt with later in greater detail.

The relative efficacy of elementary bodies and the "large forms" as immunizing agents.

It will be recalled that the virus of psittacosis shows considerable variation in size and that the large forms of the virus are those which appear in the early stages of multiplication (Bedson and Bland, 1932). The transition from large form through those intermediate in size to the small elementary body is of regular occurrence during multiplication and it has been suggested that the large form is the vegetative form of the virus, whereas the elementary body represents a resistant and resting stage of the virus adapted to withstand those extracellular conditions encountered during transmission of infection (Bedson and Bland, 1934). The immunity conferred by infection with a virus is much more solid than that produced by the injection of a formolized vaccine; sometimes the disparity is very great. Amongst those factors responsible for this difference it has been suggested that the virus present in the material from which the vaccines are made might lack some important antigen only elaborated during active multiplication and lost later when the intracellular virus colony has reached maturity and the cell is ready to break down and liberate its virus content. Should this be so, and should the large form of the psittacosis virus represent the vegetative phase of this virus, then a formolized vaccine made from virus in the large form might be a more effective immunizing agent than one made from elementary bodies. An experiment was made to see whether this were so. Suspensions were made from the spleens of mice killed 16 to 17 hours after infection and of mice dying or moribund 48 hours after infection; in the former preparation the virus was almost entirely composed of large forms. The suspensions were titrated for virus content in the guinea-pig's skin and then adjusted so as to contain approximately the same amount of virus. This entailed considerable dilution of the elementary body suspension, and in order to keep the tissue protein content of the two vaccines comparable a 5 per cent. suspension of normal mouse spleen was used as diluent. Both suspensions were formolized in the usual way and mice were immunized with the vaccines according to the plan of the previous experiments. Ten days after the last dose of vaccine the mice were tested for immunity, with the result given in Table III.

TABLE III.—*Comparison of Immunizing Power of Vaccines Made from Large Forms and Elementary Bodies.*

Test dose of virus. 0.5 c.c. intraperitoneally of a 5 per cent. virulent mouse spleen suspension diluted:	Mice immunized with— Large form vaccine.		Elementary body vaccine.		*Nil.*	
10^{-1}	D. 5	D. 8	D. 52	D. 59	D. 3	D. 5
10^{-2}	D. 6	D. 6	D. 8	D. 21	D. 5	D. 5
10^{-3}	D. 11	D. 12	D. 24	D. 27	D. 6	D. 6
10^{-4}	D. 10	D. 14	D. 10	D. 10	D. 8	D. 8
10^{-5}	S.	S.	D. 43	S.	D. 7	D. 11
10^{-6}	S.	S.	S.	S.	D. 11	D. 22

In view of the low virus content of the vaccines it was not expected that a high degree of immunity would be obtained unless the large forms proved to

be definitely superior to elementary bodies as antigens. With both vaccines, however, only a low degree of immunity was obtained.

Duration of the immunity produced by formolized vaccines.

So far the test of immunity had been applied a few days—usually 10—after the last dose of vaccine ; no attempt had been made to find out whether the immunity evoked by formol-inactivated virus was a durable one. In the following experiment 12 mice which had been immunized with formolized vaccine were left for longer periods of time before being subjected to a test of immunity. At the time of the first test, 51 days after the last dose of vaccine, 4 of the mice were killed and examined for the presence of virus in the manner described above, with a negative result. The remaining 4 mice were left for a further 58 days—109 days after the last injection of vaccine—before being given the test injection of living virus. As will be seen from Table IV, all the vaccinated mice survived a dose of virus which killed normal mice in 6–7 days, showing that the protection obtained by means of formolized virus is not merely a short-lived one.

TABLE IV.—*Duration of Immunity Produced by a Formolized Vaccine.*

Interval between last dose of vaccine and test of immunity.	Test-dose of virus.	Result.	
		Vaccinated mice.	Control mice.
51 days	>100<1000	S. S. S. S.	D. 6 D. 7
109 ,,	M.L.D. intraperitoneally	S. S. S. S.	D. 7 D. 7

The fate of virus injected into immune mice.

Earlier in this paper reference was made to the observation that mice which had been immunized with a formolized vaccine became infected when inoculated with live virus, even though they showed no symptoms of disease as the result of the test injection. In such mice, killed two or three months after the test of immunity, it has always been possible to demonstrate virus in the spleen by mouse inoculation, and in some instances virus has been present in such quantity as to be demonstrable microscopically. These findings suggested that in the immune mice, despite the absence of symptoms following the injection of live virus, the virus actually multiplied and established itself in the tissues of the immune animal. Proof of this is to be found in the following experiment in which the vaccinated mice and normal mice were injected peritoneally with between 100 and 1000 M.L.D. and then, 2, 4 and 7 days later, a mouse in each group was killed and the presence of virus in the spleen and heart's blood determined by mouse inoculation and the amount present in the former situation estimated by titration in the guinea-pig's skin. The findings are given in Table V.

It is quite obvious from these results that multiplication of virus does actually take place in the immune mice and that very small quantities of virus even appear in the blood stream. This multiplication, however, stops short of that which occurs in the normal animal. The immunized mice showed no

obvious signs of illness. In saying this the word " obvious " is used to qualify the statement as all these mice were housed in glass jars, two to a jar, and under such conditions it is impossible to be certain whether or not they are showing slight signs of illness. However, they were not obviously ill, whereas the normal mice from the fourth day onwards all were. It would appear then that the effect of immunization with a formolized vaccine is to convert an apparent and fatal infection into an inapparent one. This change might be brought about either by the multiplication of virus being hindered to such an extent that it never reached that quantity necessary for the production of frank disease or there might, in addition, be some qualitative change in the virus, a reduction in virulence. Two facts speak in favour of the former and simpler hypothesis. In a second experiment, similar to that recorded in Table V, the mice killed on the 3rd, 5th and 7th days after injection of the virus were examined not only for the presence of virus in the spleen and heart's blood, but also for any histological change in the liver and spleen. It is in these organs that one finds the most manifest and important changes in psittacosis in the mouse ; they have been described in detail by Rivers and Berry (1931) and comprise congestion, hæmorrhage and focal necrosis, with leucocytic infiltration. The differences between the histological picture presented by the spleens and livers of the immunized mice and of the normal mice was one of degree. In both the same changes could be made out but they were very much more extensive and advanced in the case of the normal mice. Thus, in the case of two mice from the normal group which were killed when moribund on the 7th day the sections of liver showed a well-marked perihepatitis, congestion and several small hæmorrhages and numerous areas of necrosis, whereas the preparations made from the livers of two mice in the immune group killed at the same time showed an absence of periphepatitis, little congestion and a few small necrotic foci. The interesting point, however, is that these changes did actually occur in the immune mice and their occurrence hardly supports the view that the virus multiplying in the immune mice suffers any reduction in virulence.

The effect on the virus of serial passage in immune mice.

The other evidence which is opposed to any qualitative change in the virus growing in the immune animal was obtained from an experiment, continued for the greater part of a year, in which virus was passed serially in immune mice. A highly virulent mouse-passage strain of virus was inoculated intraperitoneally into immunized and normal mice in a dose which killed the normal animals in 7 to 8 days. Seven days after inoculation one of the immune mice was killed and a suspension made from its spleen in M/50 phosphate. This suspension was used to inoculate further immunized and normal mice, the spleens of the immune mice being used 89 days later for a similar transfer. In this way five passages were made in immune mice, the virus being 290 days in all in this environment. At every passage direct microscopical examination was made of spleen smears so as to get a rough idea of the amount of virus present. The results recorded in Table VI give no suggestion of any change in virulence on the part of the virus for the 5 per cent. spleen suspensions made

TABLE V.—*Multiplication of Virus in Actively Immunized Mice.*

Mouse.	Treatment.	Test-dose of virus.	Time after injection of virus.	Virus content of spleen and heart's blood.					
				Spleen (5 per cent. suspension).					Heart's blood (mice injected intraperitoneally with 0·05 c.c.).
				Injection of mice (0·5 c.c. intraperitoneally).		Titration in guinea-pig's skin (0·2 c.c. intradermally diluted).			
						1/1.	1/10.	1/100. 1/1000.	
40	3 doses of vaccine 0·3 c.c. each intraperitoneally	>100	2 days	D. 8	D. 12	+	−	− −	S. D. 24
41			4 „	D. 10	S.	+	∓	− −	S. D. 9
42			7 „	D. 6	D. 7	+	±	∓ −	S. D. 21
43–47		<1000	Remained well.						
64	*Nil*	M.L.D. intraperitoneally	2 days	D. 5	D. 7	++	+	− −	S. S.
65			4 „	D. 4	D. 5	++	+	± −	D. 9 D. 10
66			7 „	D. 3	D. 4	++	++	+ ±	D. 8 D. 8
67–69			2 died (7th and 8th day); 1 recovered after appearing moribund.						

TABLE VI.—*Effect on Virus of Serial Transfer in Immune Mice.*

Generation in immune mice.		Nature and size of inoculum.			Mice immunized with formolized vaccine.	Normal mice.
No.	Duration in immune mice.	Origin.	Presence of virus in spleen smear (microscope).	Amount of 5 per cent. spleen suspension injected intraperitoneally.		
1	..	322nd passage in normal mice	−	$0·5 \times 10^{-4}$ c.c.	S. S. S. S. D. 10	D. 7 D. 8 D. 8
2	7 days	Survivor from 1st generation	+ small amount	0·5 c.c.	S. S. S.	D. 7 D. 8
3	89 „	Survivors from 2nd generation	? +	0·5 c.c.	S. S. D. 33	D. 9 D. 9
4	57 „	Survivors from 3rd generation	? +	0·5 c.c.	S. S.	D. 9 D. 9
5	137 „	Survivors from 4th generation	? +	0·5 c.c.	S. S.	D. 9 D. 10

from the different passages killed normal mice in approximately the same time and the spleen smears showed no obvious increase in the amount of virus present.

Duration of the infection in immune mice.

So far the majority of immunized mice had been killed and examined for persistence of infection some 2 or 3 months after the test injection of virus; in the last experiment 2 had been found infected after an interval of 4 months. In order to obtain some idea of the length of time for which such an infection may persist, a number of mice which had been immunized with formolized vaccine were injected with between 100 and 1000 M.L.D. of virus intraperitoneally and then set aside to be killed, two at a time, at increasing intervals of time and examined for the presence of virus in their spleens. Each spleen was examined separately. Smears stained by Giemsa and Castañeda were examined microscopically and a 5 per cent. suspension of the spleen in M/50 phosphate buffer was injected intraperitoneally into two normal mice in a dose of 0·5 c.c. Any mice surviving such an injection were re-injected with a dose of virus of between 100 and 1000 M.L.D. to determine whether they had had a subclinical infection. The results of this experiment given in Table VII show that infection persisted for as long as 7 months, but not as long as 9 months. It is not improbable that the repetition of such an experiment over a longer period of time and with a greater number of animals would show that in individual mice infection would persist for even longer.

TABLE VII.—*Persistence of Infection after Injection of Virus in Actively Immunized Mice (Formolized Vaccine).*

Interval between injection of virus and examination for infection.	Number examined.	Presence of virus in spleen. Microscopic examination. Castañeda.	Giemsa.	Mouse inoculation (5 per cent. spleen suspension, 0·5 c.c. intraperitoneally).		Retest of suvivors (>100<1000 M.L.D. intraperitoneally).	
3 months	2	? +	? +	D. 8	D. 8	..	..
		? +	?	D. 8	D. 8	..	..
5 „	2	? +	?	D. 11	D. 11	..	..
		?	—	S.	S.	D. 8	D. 9
7 „	2	?	?	D. 8	D. 11	..	..
		—	—	D. 11	S.	..	S.
9 „	2	—	—	S.	S.	D. 6	D. 7
		—	—	S.	S.	D. 3	D. 7

The mechanism of the experimental immunity in mice.

From time to time in the past it has been suggested that the immunity to some viruses, at any rate, is dependent partly, if not entirely, on a change in the susceptible cells, a tissue immunity in other words. The experiments of Andrewes (1929, 1930) with herpes, vaccinia and virus III are not in keeping with this view, since he was able to cultivate these viruses *in vitro* with tissue derived from immune animals provided these tissues were adequately washed. It might be argued, however, that the conditions pertaining in tissue culture were so artificial as to alter the behaviour of the cells and make them susceptible

of sustaining the growth of a virus which they would have resisted in their normal setting *in vivo*. In the experiments reported in this paper this argument is not valid, since it has been shown that in the immune animal psittacosis virus is able to establish itself intracellularly and multiply, though that multiplication stops short of what is necessary for the production of frank disease. It would seem, then, that the immunity to this virus evoked by formolized vaccine is not dependent on any specific change in the susceptible cells and one is forced to conclude that it is a function of antibody.

Rôle of specific antibody in immunity to psittacosis.

At the commencement of this paper reference was made to the work of Rivers and Schwentker (1934) demonstrating that either as the result of infection or immunization with living virus introduced by a non-infecting route (intramuscular) neutralizing antibodies appear in the circulation in both man and the monkey. It has also been shown (Bedson, 1933) that guinea-pigs which had recovered from infection and had been given a series of intraperitoneal injections of live virus developed neutralizing antibodies. In no case, however, has the protective titre been of a high order; in fact, prior to the elaboration of a more delicate test for the demonstration of neutralizing antibody (Rivers and Schwentker, 1934), doubt had been expressed as to their existence in human convalescent serum (Bedson and Western, 1930; Rivers, Berry and Rhoads, 1930). That mice which are immune and are carrying the infection possess some neutralizing antibody in their circulation is shown by the experiment, recorded in Table VIII, in which mice which had been immunized with a formol vaccine and resisted a test with live virus, were tested for persistence of infection and circulating antibody. At the same time similar survivors were given a severe test of immunity by the intraperitoneal injection of approximately 10,000 M.L.D. It is clear that the pooled serum of the survivors had some neutralizing antibody in their serum. Three out of four similar survivors resisted the intraperitoneal injection of a large amount of virus. It is interesting to note that in the 4 survivors furnishing the pooled serum virus was demonstrable by means of the microscope in the spleen.

In another experiment in which the pooled serum from 8 survivors from the same batch of mice was used, a similar result was obtained. In this case the pooled serum was mixed with virus so as to give a final virus concentration of approximately 1000 M.L.D. in 0·5 c.c. of the mixture. A control preparation containing normal mouse serum in place of that from the immune survivors was also made, and after standing at room temperature for 2 hours these mixtures were injected intraperitoneally in mice, the control mixture in a dose of 0·1 c.c. and that containing the immune serum in doses of 0·1 c.c. and 0·5 c.c. and 1·0 c.c., 4 mice to each dose. As will be seen from the results given in Table IX, the serum from the immune survivors contains some small degree of neutralizing power. They also show the importance of the size of dose injected in such a neutralization experiment, as it is only in the case of the 0·5 c.c. dose that evidence of neutralization is obtained. Findlay (1936) has already drawn attention to this phenomenon and pointed out that it is another way of demonstrating the dilution phenomenon in which apparently

TABLE VIII.—*Presence of Circulating Antibody in Actively Immunized Mice.*

Mouse.	Previous treatment.	Presence of virus in spleen.		Test for antibody in serum.								Test of immunity (intraperitoneal injection of approximately 10,000 M.L.D.).
		Microscope (Castañeda or Giemsa preparation).	Mouse inoculation (5 per cent. spleen suspension; 2 mice, 0·5 c.c. intraperitoneally).	Pooled serum A, B, C, D, mixed undiluted with virus to give approximately 10,000 M.L.D. per 0.5 c.c. Injected after 2 hours at room temperature to 4 mice 0·5 c.c. intraperitoneally.				Control mixture similarly made, but with normal mouse serum.				
A	3 doses of formol vaccine. Survived intraperitoneal injection of 10–100 M.L.D. Examined after 2 months	+	D. 10 D. 10	D. 15	D. 16	S.	S.	D. 7	D. 7	D. 9	D. 9	D. 6
B		+	D. 7 D. 10									S.
C		+	D. 10 D. 12									S.
D		+	D. 9 D. 12									S.
E												D. 6
F												D. 6
G												D. 7
H												D. 7
N1	*Nil.*											
N2												
N3												
N4												

neutral mixtures of serum and virus are rendered virulent by dilution, the size of the inoculum being kept constant. Presumably in the case of the mice receiving 1·0 c.c. of the mixture, although they got more serum, this was insufficient passively to immunize them against the greater amount of virus injected.

TABLE IX.—*Presence of Circulating Antibody in Actively Immunized Mice.*

Serum-virus mixture.	Dose injected.	Result (4 mice).			
Pooled serum from 8 mice which had received formol vaccine and survived a test with live virus; bled 2 months later.	1·0 c.c. .	D. 8	D. 10	D. 11	D. 22
	0·5 c.c. .	D. 20	S.	S.	S.
Mixed undiluted with equal parts of virus suspension to give approximately 1000 M.L.D. per 0·5 c.c of mixture. 2 hours room temperature.	0·1 c.c. .	D. 9	D. 10	D. 11	D. 12
Similar mixture, but made with normal mouse serum. 2 hours room temperature.	0·1 c.c. .	D. 7	D. 10	D. 10	D. 10

It is clear, then, that mice immune to the extent of showing no symptoms of disease when injected with psittacosis virus, do possess some neutralizing antibody in their circulation. Over a restricted range of virus dosage it is possible to demonstrate passive immunity against psittacosis virus, and in view of the evidence which has been produced it is reasonable to suppose that the mechanism of the immunity possessed by mice which have received formolized vaccine is solely a function of antibody. This is made all the more likely by the fact that mice which have supported, without obvious illness, the injection of virus under cover of passive immunity, become infected just as do the vaccinated mice and develop a solid immunity as the result of this infection.

TABLE X.—*Persistence of Infection after Injection of Recently-Made Serum-Virus Mixtures.*

Mouse.	Serum-virus injection.	Subsequent test of immunity. When.	Subsequent test of immunity. Dose of virus.	Subsequent test of immunity. Result.	Presence of virus in spleen. Time after last inoculation of virus.	Presence of virus in spleen. Microscopic examination.	Presence of virus in spleen. Mouse inoculation.
A.	Received various serum-virus mixtures intraperitoneally showed no signs of illness	After 2 months	10,000 M.L.D. intraperitoneally	S.	1 month	+	+
B.				S.		−	+
C.				S.		−	+
D.				S.		−	+
E.				S.		−	+
F.			None		3 months	−	+
G.						−	+
N 1.	None		10,000 M.L.D. intraperitoneally	D. 4			
N. 2				D. 4			

In Table X are recorded the findings in mice which had received recently-made mixtures of antiserum and virus in which union of virus and antibody had not yet had time to take place. Some of the mice were tested for immunity after a lapse of 2 months, and a month later were examined for persistent

infection, others were examined for presence of virus in their tissues 3 months after having been given the serum-virus mixture. In these mice the picture found was similar to that seen in the actively immunized mice.

DISCUSSION.

The evidence produced in this paper leaves little doubt that a considerable degree of immunity can be produced in mice by the injection of formolized psittacosis virus. And the fact that it has not been found possible to recover virus from the spleens of these vaccinated mice, even by three serial passages in normal mice, does suggest that the virus in these vaccines was dead and that their efficacy was not due to their ability to infect. Had there been a small amount of virus in these vaccines which had remained unaffected by the formalin one would have expected serial passage with the spleens of the vaccinated animals to have revealed it. On the other hand, had the action of the formalin merely been to alter or attenuate the virus without killing it, so that it still multiplied without producing disease when injected into mice, it should have been possible to detect it microscopically, at any rate occasionally. There is evidence that other viruses will immunize when completely inactivated and to these must now be added psittacosis virus. The surprising thing is not that immunity can be produced with killed psittacosis virus, but that the immunity so produced is a relatively good and durable one. And in this respect it might be pointed out that the results obtained do not necessarily represent the best that could be achieved, for it is possible that by alterations in the method of formolization and paying more attention to the spacing of the doses of vaccine, a more satisfactory immunity could be obtained. However, satisfactory though the immunity produced by a formol vaccine may appear to be, it does not protect the animal completely, even against small doses of virus. Infection has been the rule when the test of immunity has been applied, but unless the dose of virus injected has been a big one, multiplication has been kept below the threshold necessary for the production of frank disease. And this infection, once established, has persisted up to 7 months, which is a considerable time in the life of a mouse. Whether, during the course of this symptomless infection, the balance between host and parasite might be upset and frank disease result has not been determined. Attempts have been made to break down the immunity of vaccinated mice by giving a large dose of vaccine two or three hours prior to the test dose of live virus, the idea being that the antigen in the vaccine might neutralize specific antibody and so remove the barrier preventing virus reaching the susceptible cells; these attempts have been unsuccessful. This line of investigation has, however, been insufficiently explored, and since, in the author's experience, birds sometimes relapse and die of psittacosis after an apparent recovery from a natural attack of the disease, it is probable that the same might occur occasionally in the case of the silent experimental infections in the mouse. Even though the non-infective vaccine gives quite a good immunity, there is no doubt that actual infection gives a better one. Mice that have been immunized with formolized vaccine and then given a sub-clinical infection are more solidly immune than those which have only received the vaccine. Whether the effect

of the infection is to produce some change in the susceptible cells or merely to reinforce antibody production it is difficult to say; possibly the latter explanation is the correct one. But the fact that infection produces a more solid immunity than the injection of a dead vaccine is in no way surprising, for the same is probably true of the immunity to any micro-organism.

If, then, one attempts to form some idea of the mechanism of the immunity evoked by a natural attack of psittacosis, bearing in mind the additional fact that in birds recovery from psittacosis frequently, if not invariably, leaves behind it a residual infection, ones conception would probably be as follows: In those cases which are going to recover the gradual increase of antibody in the circulation and tissue spaces would render the infection of fresh cells increasingly difficult until, with the onset of convalescence, infection would be confined to only a few cells. From time to time some of these remaining infected cells, killed by the virus growing in them, would break down, liberating virus most of which, meeting and uniting with antibody, would be prevented either from entering fresh susceptible cells or, having done so, from multiplying. A few virus particles, however, escaping the antibody would enter and infect new cells, thus perpetuating the infection. At the same time these intermittent showers of virus would provide the antigenic stimuli necessary to maintain antibody at an effective level. The duration of such a carrier state, depending as it would on the chance infection of occasional cells, would be variable and should the residual infection die out the immunity would gradually wane and the individual become once more susceptible. This conception of the immunity to psittacosis would place it midway between "premunition" and immunity, as defined by Sergent and Parrot (1935) in that, although improved by persistence of infection, it would not appear to depend entirely on this and can be produced by means of a dead vaccine. It is not suggested that the mechanism of immunity to psittacosis virus differs in any fundamental manner from the immunity to the other viruses, for it seems highly probable that an immunity mechanism such as this would occur in the case of any micro-organism adapted to an intracellular mode of life.

CONCLUSIONS.

1. Immunity can be produced in mice to psittacosis virus by means of a formolized vaccine in which no living virus is demonstrable.

2. This immunity lasts for at least three months.

3. The protection so obtained is not complete, even against small doses of virus, since the test injection of active virus almost invariably results in infection. Multiplication of the virus, however, stops short of that required for the production of frank disease, the infection remaining silent.

4. Silent infection in vaccinated mice has been found to last for as long as 7 months.

5. Serial passage of virus in immunized mice through 5 generations and extending over a period of 290 days produced no appreciable change in the virulence of the virus.

6. Specific antibody in low concentration can be demonstrated by the neutralization test in the circulation of immune mice.

7. The injection of mice with an apparently neutral serum-virus mixture results in a silent infection, which, as in the case of the actively immunized mice, may last for months.

8. The mechanism of immunity to psittacosis virus is discussed and it is suggested that it is principally a function of specific antibody.

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